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<b>(54) Title:</b> MULTIVALENT VACCINE FOR <i>CLOSTRIDIUM BOTULINUM</i> NEUROTOXIN		
<b>(57) Abstract</b>  The present invention includes recombinant proteins derived from <i>Clostridium botulinum</i> toxins. In particular, soluble recombinant <i>Clostridium botulinum</i> type A, type B and type E toxin proteins are provided. Methods which allow for the isolation of recombinant proteins free of significant endotoxin contamination are provided. The soluble, endotoxin-free recombinant proteins are used as immunogens for the production of vaccines and antitoxins. These vaccines and antitoxins are useful in the treatment of humans and other animals at risk of intoxication with clostridial toxin.		

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## MULTIVALENT VACCINE FOR *CLOSTRIDIUM BOTULINUM* NEUROTOXIN

### FIELD OF THE INVENTION

The present invention relates to the isolation of polypeptides derived from *Clostridium*  
*botulinum* neurotoxins and the use thereof as immunogens for the production of vaccines,  
 including multivalent vaccines, and antitoxins.

### BACKGROUND OF THE INVENTION

The genus *Clostridium* is comprised of gram-positive, anaerobic, spore-forming bacilli.  
 The natural habitat of these organisms is the environment and the intestinal tracts of humans  
 and other animals. Indeed, clostridia are ubiquitous: they are commonly found in soil, dust,  
 sewage, marine sediments, decaying vegetation, and mud. [See e.g., P.H.A. Sneath *et al.*,  
 "*Clostridium*," *Bergey's Manual of Systematic Bacteriology*, Vol. 2, pp. 1141-1200,  
 Williams & Wilkins (1986).] Despite the identification of approximately 100 species of  
*Clostridium*, only a small number have been recognized as etiologic agents of medical and  
 veterinary importance. Nonetheless, these species are associated with very serious diseases,  
 including botulism, tetanus, anaerobic cellulitis, gas gangrene, bacteremia, pseudomembranous  
 colitis, and clostridial gastroenteritis. Table I lists some of the species of medical and  
 veterinary importance and the diseases with which they are associated. As virtually all of  
 these species have been isolated from fecal samples of apparently healthy persons, some of  
 these isolates may be transient, rather than permanent residents of the colonic flora.

TABLE I

*Clostridium* Species Of Medical And Veterinary Importance\*

Species	Disease
<i>C. aminovalericum</i>	Bacteriuria (pregnant women)
<i>C. argentinense</i>	Infected wounds; Bacteremia; Botulism; Infections of amniotic fluid
<i>C. baratii</i>	Infected war wounds; Peritonitis; Infectious processes of the eye, ear and prostate
<i>C. beijerinckii</i>	Infected wounds
<i>C. bifermians</i>	Infected wounds; Abscesses; Gas Gangrene; Bacteremia
<i>C. botulinum</i>	Food poisoning; Botulism (wound, food, infant)
<i>C. butyricum</i>	Urinary tract, lower respiratory tract, pleural cavity, and abdominal infections; Infected wounds; Abscesses; Bacteremia
<i>C. cadaveris</i>	Abscesses; Infected wounds

TABLE I

*Clostridium* Species Of Medical And Veterinary Importance\*

Species	Disease
<i>C. carnis</i>	Soft tissue infections: Bacteremia
<i>C. chauvoei</i>	Blackleg
<i>C. clostridioforme</i>	Abdominal, cervical, scrotal, pleural, and other infections; Septicemia; Peritonitis; Appendicitis
<i>C. cochlearium</i>	Isolated from human disease processes, but role in disease unknown.
<i>C. difficile</i>	Antimicrobial-associated diarrhea; Pseudomembranous enterocolitis; Bacteremia; Pyogenic infections
<i>C. fallax</i>	Soft tissue infections
<i>C. ghonii</i>	Soft tissue infections
<i>C. glycolicum</i>	Wound infections; Abscesses; Peritonitis
<i>C. hastiforme</i>	Infected war wounds; Bacteremia; Abscesses
<i>C. histolyticum</i>	Infected war wounds; Gas gangrene; Gingival plaque isolate
<i>C. indolis</i>	Gastrointestinal tract infections
<i>C. innocuum</i>	Gastrointestinal tract infections; Empyema
<i>C. irregulare</i>	Penile lesions
<i>C. leptum</i>	Isolated from human disease processes, but role in disease unknown.
<i>C. limosum</i>	Bacteremia; Peritonitis; Pulmonary infections
<i>C. malenominatum</i>	Various infectious processes
<i>C. novyi</i>	Infected wounds; Gas gangrene; Blackleg; Big head (ovine); Redwater disease (bovine)
<i>C. oroticum</i>	Urinary tract infections; Rectal abscesses
<i>C. paraputrificum</i>	Bacteremia; Peritonitis; Infected wounds; Appendicitis
<i>C. perfringens</i>	Gas gangrene; Anaerobic cellulitis; Intra-abdominal abscesses; Soft tissue infections; Food poisoning; Necrotizing pneumonia; Empyema; Meningitis; Bacteremia; Uterine Infections; Enteritis necrotans; Lamb dysentery; Struck; Ovine Enterotoxemia;
<i>C. putrefaciens</i>	Bacteriuria (Pregnant women with bacteremia)
<i>C. putrificum</i>	Abscesses; Infected wounds; Bacteremia
<i>C. ramosum</i>	Infections of the abdominal cavity, genital tract, lung, and biliary tract; Bacteremia
<i>C. sartagoforme</i>	Isolated from human disease processes, but role in disease unknown.
<i>C. septicum</i>	Gas gangrene; Bacteremia; Suppurative infections; Necrotizing enterocolitis; Braxy
<i>C. sordellii</i>	Gas gangrene; Wound infections; Penile lesions; Bacteremia; Abscesses; Abdominal and vaginal infections



TABLE I

*Clostridium* Species Of Medical And Veterinary Importance\*

Species	Disease
<i>C. sphenoides</i>	Appendicitis; Bacteremia; Bone and soft tissue infections; Intraperitoneal infections; Infected war wounds; Visceral gas gangrene; Renal abscesses
<i>C. sporogenes</i>	Gas gangrene; Bacteremia; Endocarditis; central nervous system and pleuropulmonary infections; Penile lesions; Infected war wounds; Other pyogenic infections
<i>C. subterminale</i>	Bacteremia; Empyema; Biliary tract, soft tissue and bone infections
<i>C. symbiosum</i>	Liver abscesses; Bacteremia; Infections resulting due to bowel flora
<i>C. tertium</i>	Gas gangrene; Appendicitis; Brain abscesses; Intestinal tract and soft tissue infections; Infected war wounds; Periodontitis; Bacteremia
<i>C. tetani</i>	Tetanus; Infected gums and teeth; Corneal ulcerations; Mastoid and middle ear infections; Intraperitoneal infections; Tetanus neonatorum; Postpartum uterine infections; Soft tissue infections, especially related to trauma (including abrasions and lacerations); Infections related to use of contaminated needles
<i>C. thermosaccharolyticum</i>	Isolated from human disease processes, but role in disease unknown.

- \* Compiled from P.G. Engelkirk *et al.* "Classification", *Principles and Practice of Clinical Anaerobic Bacteriology*, pp. 22-23, Star Publishing Co., Belmont, CA (1992); J. Stephen and R.A. Petrowski, "Toxins Which Traverse Membranes and Deregulate Cells," in *Bacterial Toxins*, 2d ed., pp. 66-67, American Society for Microbiology (1986); R. Berkow and A.J. Fletcher (eds.), "Bacterial Diseases," *Merck Manual of Diagnosis and Therapy*, 16th ed., pp. 116-126, Merck Research Laboratories, Rahway, N.J. (1992); and O.H. Sigmund and C.M. Fraser (eds.), "Clostridial Infections," *Merck Veterinary Manual*, 5th ed., pp. 396-409, Merck & Co., Rahway, N.J. (1979).

In most cases, the pathogenicity of these organisms is related to the release of powerful exotoxins or highly destructive enzymes. Indeed, several species of the genus *Clostridium* produce toxins and other enzymes of great medical and veterinary significance. [C.L. Hatheway, Clin. Microbiol. Rev. 3:66-98 (1990).]

Perhaps because of their significance for human and veterinary medicine, much research has been conducted on these toxins, in particular those of *C. botulinum* and *C. difficile*.

### *C. botulinum*

Several strains of *Clostridium botulinum* produce toxins of significance to human and animal health. [C.L. Hatheway, Clin. Microbiol. Rev. 3:66-98 (1990)] The effects of these toxins range from diarrheal diseases that can cause destruction of the colon, to paralytic effects that can cause death. Particularly at risk for developing clostridial diseases are

neonates and humans and animals in poor health (e.g., those suffering from diseases associated with old age or immunodeficiency diseases).

*Clostridium botulinum* produces the most poisonous biological toxin known. The lethal human dose is a mere  $10^{-9}$  mg/kg bodyweight for toxin in the bloodstream. Botulinal toxin blocks nerve transmission to the muscles, resulting in flaccid paralysis. When the toxin reaches airway and respiratory muscles, it results in respiratory failure that can cause death. [S. Arnon, J. Infect. Dis. 154:201-206 (1986)]

*C. botulinum* spores are carried by dust and are found on vegetables taken from the soil, on fresh fruits, and on agricultural products such as honey. Under conditions favorable to the organism, the spores germinate to vegetative cells which produces toxin. [S. Arnon, Ann. Rev. Med. 31:541 (1980)]

Botulism disease may be grouped into four types, based on the method of introduction of toxin into the bloodstream. Food-borne botulism results from ingesting improperly preserved and inadequately heated food that contains botulinal toxin. There were 355 cases of food-borne botulism in the United States between 1976 and 1984. [K.L. MacDonald *et al.*, Am. J. Epidemiol. 124:794 (1986).] The death rate due to botulinal toxin is 12% and can be higher in particular risk groups. [C.O. Tacket *et al.*, Am. J. Med. 76:794 (1984).] Wound-induced botulism results from *C. botulinum* penetrating traumatized tissue and producing toxin that is absorbed into the bloodstream. Since 1950, thirty cases of wound botulism have been reported. [M.N. Swartz, "Anaerobic Spore-Forming Bacilli: The Clostridia," pp. 633-646, in B.D. Davis *et al.* (eds.), *Microbiology*, 4th edition, J.B. Lippincott Co. (1990).] Inhalation botulism results when the toxin is inhaled. Inhalation botulism has been reported as the result of accidental exposure in the laboratory [E. Holzer, Med. Klin. 41:1735 (1962)] and could arise if the toxin is used as an agent of biological warfare [D.R. Franz *et al.*, in *Botulinum and Tetanus Neurotoxins*, B.R. DasGupta, ed., Plenum Press, New York (1993), pp. 473-476]. Infectious infant botulism results from *C. botulinum* colonization of the infant intestine with production of toxin and its absorption into the bloodstream. It is likely that the bacterium gains entry when spores are ingested and subsequently germinate. [S. Arnon, J. Infect. Dis. 154:201 (1986).] There have been 500 cases reported since it was first recognized in 1976. [M.N. Swartz, *supra*.]

Infant botulism strikes infants who are three weeks to eleven months old (greater than 90% of the cases are infants less than six months). [S. Arnon, J. Infect. Dis. 154:201 (1986).] It is believed that infants are susceptible, due, in large part, to the absence of the full adult complement of intestinal microflora. The benign microflora present in the adult intestine provide an acidic environment that is not favorable to colonization by *C. botulinum*. Infants begin life with a sterile intestine which is gradually colonized by microflora. Because of the limited microflora present in early infancy, the intestinal environment is not as acidic, allowing for *C. botulinum* spore germination, growth, and toxin production. In this regard, some adults who have undergone antibiotic therapy which alters intestinal microflora become more susceptible to botulism.

An additional factor accounting for infant susceptibility to infectious botulism is the immaturity of the infant immune system. The mature immune system is sensitized to bacterial antigens and produces protective antibodies. Secretory IgA produced in the adult intestine has the ability to agglutinate vegetative cells of *C. botulinum*. [S. Arnon, J. Infect. Dis. 154:201 (1986).] Secretory IgA may also act by preventing intestinal bacteria and their products from crossing the cells of the intestine. [S. Arnon, Epidemiol. Rev. 3:45 (1981).] The infant immune system is not primed to do this.

Clinical symptoms of infant botulism range from mild paralysis, to moderate and severe paralysis requiring hospitalization, to fulminant paralysis, leading to sudden death. [S. Arnon, Epidemiol. Rev. 3:45 (1981).]

The chief therapy for severe infant botulism is ventilatory assistance using a mechanical respirator and concurrent elimination of toxin and bacteria using cathartics, enemas, and gastric lavage. There were 68 hospitalizations in California for infant botulism in a single year with a total cost of over \$4 million for treatment. [T.L. Frankovich and S. Arnon, West. J. Med. 154:103 (1991).]

Different strains of *Clostridium botulinum* each produce antigenically distinct toxin designated by the letters A-G. Serotype A toxin has been implicated in 26% of the cases of food botulism; types B, E and F have also been implicated in a smaller percentage of the food botulism cases [H. Sugiyama, Microbiol. Rev. 44:419 (1980)]. Wound botulism has been reportedly caused by only types A or B toxins [H. Sugiyama, *supra*]. Nearly all cases of infant botulism have been caused by bacteria producing either type A or type B toxin.

(Exceptionally, one New Mexico case was caused by *Clostridium botulinum* producing type F toxin and another by *Clostridium botulinum* producing a type B-type F hybrid.) [S. Arnon, Epidemiol. Rev. 3:45 (1981).] Type C toxin affects waterfowl, cattle, horses and mink. Type D toxin affects cattle, and type E toxin affects both humans and birds.

5 A trivalent antitoxin derived from horse plasma is commercially available from Connaught Industries Ltd. as a therapy for toxin types A, B, and E. However, the antitoxin has several disadvantages. First, extremely large dosages must be injected intravenously and/or intramuscularly. Second, the antitoxin has serious side effects such as acute anaphylaxis which can lead to death, and serum sickness. Finally, the efficacy of the  
10 antitoxin is uncertain and the treatment is costly. [C.O. Tacket *et al.*, Am. J. Med. 76:794 (1984).]

A heptavalent equine botulinal antitoxin which uses only the F(ab')<sub>2</sub> portion of the antibody molecule has been tested by the United States Military. [M. Balady, USAMRDC Newsletter, p. 6 (1991).] This was raised against impure toxoids in those large animals and is  
15 not a high titer preparation.

A pentavalent human antitoxin has been collected from immunized human subjects for use as a treatment for infant botulism. The supply of this antitoxin is limited and cannot be expected to meet the needs of all individuals stricken with botulism disease. In addition, collection of human sera must involve screening out HIV and other potentially serious human  
20 pathogens. [P.J. Schwarz and S.S. Arnon, Western J. Med. 156:197 (1992).]

Infant botulism has been implicated as the cause of mortality in some cases of Sudden Infant Death Syndrome (SIDS, also known as crib death). SIDS is officially recognized as infant death that is sudden and unexpected and that remained unexplained despite complete post-mortem examination. The link of SIDS to infant botulism came when fecal or blood  
25 specimens taken at autopsy from SIDS infants were found to contain *C. botulinum* organisms and/or toxin in 3-4% of cases analyzed. [D.R. Peterson *et al.*, Rev. Infect. Dis. 1:630 (1979).] In contrast, only 1 of 160 healthy infants (0.6%) had *C. botulinum* organisms in the feces and no botulinal toxin. (S. Arnon *et al.*, Lancet, pp. 1273-76, June 17, 1978.)

In developed countries, SIDS is the number one cause of death in children between  
30 one month and one year old. (S. Arnon *et al.*, Lancet, pp. 1273-77, June 17, 1978.) More children die from SIDS in the first year than from any other single cause of death in the first

fourteen years of life. In the United States, there are 8,000-10,000 SIDS victims annually.  
*Id.*

What is needed is an effective therapy against infant botulism that is free of dangerous side effects, is available in large supply at a reasonable price, and can be safely and gently delivered so that prophylactic application to infants is feasible.

Immunization of subjects with toxin preparations has been done in an attempt to induce immunity against botulinal toxins. A *C. botulinum* vaccine comprising chemically inactivated (*i.e.*, formaldehyde-treated) type A, B, C, D and E toxin is commercially available for human usage. However, this vaccine preparation has several disadvantages. First, the efficacy of this vaccine is variable (in particular, only 78% of recipients produce protective levels of anti-type B antibodies following administration of the primary series). Second, immunization is painful (deep subcutaneous inoculation is required for administration), with adverse reactions being common (moderate to severe local reactions occur in approximately 6% of recipients upon initial injection; this number rises to approximately 11% of individuals who receive booster injections) [Informational Brochure for the Pentavalent (ABCDE) Botulinum Toxoid, Centers for Disease Control]. Third, preparation of the vaccine is dangerous as active toxin must be handled by laboratory workers.

What is needed are safe and effective vaccine preparations for administration to those at risk of exposure to *C. botulinum* toxins.

### *C. difficile*

*C. difficile*, an organism which gained its name due to difficulties encountered in its isolation, has recently been proven to be an etiologic agent of diarrheal disease. (Sneath *et al.*, p. 1165.). *C. difficile* is present in the gastrointestinal tract of approximately 3% of healthy adults, and 10-30% of neonates without adverse effect (Swartz, at p. 644); by other estimates, *C. difficile* is a part of the normal gastrointestinal flora of 2-10% of humans. [G.F. Brooks *et al.*, (eds.) "*Infections Caused by Anaerobic Bacteria*," Jawetz, Melnick, & Adelberg's *Medical Microbiology*, 19th ed., pp. 257-262, Appleton & Lange, San Mateo, CA (1991).] As these organisms are relatively resistant to most commonly used antimicrobials, when a patient is treated with antibiotics, the other members of the normal gastrointestinal

flora are suppressed and *C. difficile* flourishes, producing cytopathic toxins and enterotoxins. It has been found in 25% of cases of moderate diarrhea resulting from treatment with antibiotics, especially the cephalosporins, clindamycin, and ampicillin. [M.N. Swartz at 644.]

Importantly, *C. difficile* is commonly associated with nosocomial infections. The organism is often present in the hospital and nursing home environments and may be carried on the hands and clothing of hospital personnel who care for debilitated and immunocompromised patients. As many of these patients are being treated with antimicrobials or other chemotherapeutic agents, such transmission of *C. difficile* represents a significant risk factor for disease. (Engelkirk *et al.*, pp. 64-67.)

*C. difficile* is associated with a range of diarrhetic illness, ranging from diarrhea alone to marked diarrhea and necrosis of the gastrointestinal mucosa with the accumulation of inflammatory cells and fibrin, which forms a pseudomembrane in the affected area. (Brooks *et al.*) It has been found in over 95% of pseudomembranous enterocolitis cases. (Swartz, at p. 644.) This occasionally fatal disease is characterized by diarrhea, multiple small colonic plaques, and toxic megacolon. (Swartz, at p. 644.) Although stool cultures are sometimes used for diagnosis, diagnosis is best made by detection of the heat labile toxins present in fecal filtrates from patients with enterocolitis due to *C. difficile*. (Swartz, at p. 644-645; and Brooks *et al.*, at p. 260.) *C. difficile* toxins are cytotoxic for tissue/cell cultures and cause enterocolitis when injected intracecally into hamsters. (Swartz, at p. 644.)

The enterotoxicity of *C. difficile* is primarily due to the action of two toxins, designated A and B, each of approximately 300,000 in molecular weight. Both are potent cytotoxins, with toxin A possessing direct enterocytotoxic activity. [Lyerly *et al.*, Infect. Immun. 60:4633 (1992).] Unlike toxin A of *C. perfringens*, an organism rarely associated with antimicrobial-associated diarrhea, the toxin of *C. difficile* is not a spore coat constituent and is not produced during sporulation. (Swartz, at p. 644.) *C. difficile* toxin A causes hemorrhage, fluid accumulation and mucosal damage in rabbit ileal loops and appears to increase the uptake of toxin B by the intestinal mucosa. Toxin B does not cause intestinal fluid accumulation, but it is 1000 times more toxic than toxin A to tissue culture cells and causes membrane damage. Although both toxins induce similar cellular effects such as actin disaggregation, differences in cell specificity occurs.

Both toxins are important in disease. [Borriello *et al.*, Rev. Infect. Dis., 12(suppl. 2):S185 (1990); Lysterly *et al.*, Infect. Immun., 47:349 (1985); and Rolfe, Infect. Immun., 59:1223 (1990).] Toxin A is thought to act first by binding to brush border receptors, destroying the outer mucosal layer, then allowing toxin B to gain access to the underlying tissue. These steps in pathogenesis would indicate that the production of neutralizing antibodies against toxin A may be sufficient in the prophylactic therapy of CDAD. However, antibodies against toxin B may be a necessary additional component for an effective therapeutic against later stage colonic disease. Indeed, it has been reported that animals require antibodies to both toxin A and toxin B to be completely protected against the disease. [Kim and Rolfe, Abstr. Ann. Meet. Am. Soc. Microbiol., 69:62 (1987).]

*C. difficile* has also been reported to produce other toxins such as an enterotoxin different from toxins A and B [Banno *et al.*, Rev. Infect. Dis., 6(Suppl. 1):S11-S20 (1984)], a low molecular weight toxin [Rihn *et al.*, Biochem. Biophys. Res. Comm., 124:690-695 (1984)], a motility altering factor [Justus *et al.*, Gastroenterol., 83:836-843 (1982)], and perhaps other toxins. Regardless, *C. difficile* gastrointestinal disease is of primary concern.

It is significant that due to its resistance to most commonly used antimicrobials, *C. difficile* is associated with antimicrobial therapy with virtually all antimicrobial agents (although most commonly ampicillin, clindamycin and cephalosporins). It is also associated with disease in patients undergoing chemotherapy with such compounds as methotrexate, 5-fluorouracil, cyclophosphamide, and doxorubicin. [S.M. Finegold, *et al.*, *Clinical Guide to Anaerobic Infections*, pp. 88-89, Star Publishing Co., Belmont, CA (1992).]

Treatment of *C. difficile* disease is problematic, given the high resistance of the organism. Oral metronidazole, bacitracin and vancomycin have been reported to be effective. (Finegold *et al.*, p. 89.) However there are problems associated with treatment utilizing these compounds. Vancomycin is very expensive, some patients are unable to take oral medication, and the relapse rate is high (20-25%), although it may not occur for several weeks. *Id.*

*C. difficile* disease would be prevented or treated by neutralizing the effects of these toxins in the gastrointestinal tract. Thus, what is needed is an effective therapy against *C. difficile* toxin that is free of dangerous side effects, is available in large supply at a reasonable

price, and can be safely delivered so that prophylactic application to patients at risk of developing pseudomembranous enterocolitis can be effectively treated.

## DESCRIPTION OF THE DRAWINGS

5        Figure 1 shows the reactivity of anti-*C. botulinum* IgY by Western blot.

Figure 2 shows the IgY antibody titer to *C. botulinum* type A toxoid in eggs, measured by ELISA.

Figure 3 shows the results of *C. difficile* toxin A neutralization assays.

Figure 4 shows the results of *C. difficile* toxin B neutralization assays.

10       Figure 5 shows the results of *C. difficile* toxin B neutralization assays.

Figure 6 is a restriction map of *C. difficile* toxin A gene, showing sequences of primers 1-4 (SEQ ID NOS:1-4).

Figure 7 is a Western blot of *C. difficile* toxin A reactive protein.

Figure 8 shows *C. difficile* toxin A expression constructs.

15       Figure 9 shows *C. difficile* toxin A expression constructs.

Figure 10 shows the purification of recombinant *C. difficile* toxin A.

Figure 11 shows the results of *C. difficile* toxin A neutralization assays with antibodies reactive to recombinant toxin A.

Figure 12 shows the results for a *C. difficile* toxin A neutralization plate.

20       Figure 13 shows the results for a *C. difficile* toxin A neutralization plate.

Figure 14 shows the results of recombinant *C. difficile* toxin A neutralization assays.

Figure 15 shows *C. difficile* toxin A expression constructs.

Figure 16 shows a chromatograph plotting absorbance at 280 nm against retention time for a pMA1870-680 IgY PEG preparation.

25       Figure 17 shows two recombinant *C. difficile* toxin B expression constructs.

Figure 18 shows *C. difficile* toxin B expression constructs.

Figure 19 shows *C. difficile* toxin B expression constructs.

Figure 20 shows *C. difficile* toxin B expression constructs.

30       Figure 21 is an SDS-PAGE gel showing the purification of recombinant *C. difficile* toxin B fusion protein.



Figure 22 is an SDS-PAGE gel showing the purification of two histidine-tagged recombinant *C. difficile* toxin B proteins.

Figure 23 shows *C. difficile* toxin B expression constructs.

Figure 24 is a Western blot of *C. difficile* toxin B reactive protein.

Figure 25 shows *C. botulinum* type A toxin expression constructs; constructs used to provide *C. botulinum* or *C. difficile* sequences are also shown.

Figure 26 is an SDS-PAGE gel stained with Coomassie blue showing the purification of recombinant *C. botulinum* type A toxin fusion proteins.

Figure 27 shows *C. botulinum* type A toxin expression constructs; constructs used to provide *C. botulinum* sequences are also shown.

Figure 28 is an SDS-PAGE gel stained with Coomassie blue showing the purification of pHisBot protein using the Ni-NTA resin.

Figure 29 is an SDS-PAGE gel stained with Coomassie blue showing the expression of pHisBot protein in BL21(DE3) and BL21(DE3)pLysS host cells.

Figure 30 is an SDS-PAGE gel stained with Coomassie blue showing the purification of pHisBot protein using a batch absorption procedure.

Figure 31 is an SDS-PAGE gel stained with Coomassie blue showing the purification of pHisBot and pHisBot(native) proteins using a Ni-NTA column.

Figure 32 is an SDS-PAGE gel stained with Coomassie blue showing the purification of pHisBotA protein expressed in pHisBotA(syn) kan lacIq T7/pACYCGro/BL21(DE3) cells using an IDA column.

Figure 33 is an SDS-PAGE gel stained with Coomassie blue showing the purification of pHisBotA, pHisBotB and pHisBotE proteins by IDA chromatography followed by chromatography on S-100 to remove folding chaperones.

Figure 34 is an SDS-PAGE gel stained with Coomassie blue showing the extracts derived from pHisBotB amp T7lac/BL21(DE3) cells before and after purification on a Ni-NTA column.

Figure 35 is an SDS-PAGE gel run under native conditions and stained with Coomassie blue showing the removal of folding chaperones from IDA-purified BotB protein using a S-100 column.

Figure 36 is an SDS-PAGE gel stained with Coomassie blue showing proteins that eluted during an imidazole step gradient applied to a IDA column containing a lysate of pHisBotB kan lacIq T7/pACYCGro/BL21(DE3) cells.

Figure 37 is an SDS-PAGE gel run under native conditions and stained with Coomassie blue showing IDA-purified BotB protein before and after ultrafiltration.

Figure 38 is an SDS-PAGE gel stained with Coomassie blue showing the purification of BotE protein using a NiNTA column.

Figure 39 is an SDS-PAGE gel stained with Coomassie blue showing extracts derived from pHisBotA kan T7 lac/BL21(DE3) pLysS cells grown in fermentation culture.

Figure 40 is a chromatogram showing proteins present after IDA-purified BotE protein was applied to a S-100 column.

## DEFINITIONS

To facilitate understanding of the invention, a number of terms are defined below.

As used herein, the term "neutralizing" is used in reference to antitoxins, particularly antitoxins comprising antibodies, which have the ability to prevent the pathological actions of the toxin against which the antitoxin is directed.

As used herein, the term "overproducing" is used in reference to the production of clostridial toxin polypeptides in a host cell and indicates that the host cell is producing more of the clostridial toxin by virtue of the introduction of nucleic acid sequences encoding said clostridial toxin polypeptide than would be expressed by said host cell absent the introduction of said nucleic acid sequences. To allow ease of purification of toxin polypeptides produced in a host cell it is preferred that the host cell express or overproduce said toxin polypeptide at a level greater than 1 mg/liter of host cell culture.

"A host cell capable of expressing a recombinant protein at a level greater than or equal to 5% of the total cellular protein" is a host cell in which the recombinant protein represents at least 5% of the total cellular protein. To determine what percentage of total cellular protein the recombinant protein represents, the following steps are taken. A total of 10 OD<sub>600</sub> units of recombinant host cells (e.g., 200 µl of cells at OD<sub>600</sub> 50/ml) are removed (at a timepoint known to represent the peak of expression of the desired recombinant protein) to a 1.5 ml microfuge tube and pelleted for 2 min at maximum rpm in a microfuge. The

pellets are resuspended in 1 ml of 50 mM NaHPO<sub>4</sub>, 0.5 M NaCl, 40 mM imidazole buffer (pH 6.8) containing 1 mg/ml lysozyme. The samples are incubated for 20 min at room temperature and stored ON at -70°C. Samples are thawed completely at room temperature and sonicated 2 X 10 seconds with a Branson Sonifier 450 microtip probe at # 3 power setting. The samples are centrifuged for 5 min. at maximum rpm in a microfuge. An aliquot (20 µl) of the protein sample is removed to 20 µl 2X sample buffer (this represents the total protein extract). The samples are heated to 95°C for 5 min. then cooled and 5 or 10 µl are loaded onto 12.5% SDS-PAGE gels. High molecular weight protein markers are also loaded to allow for estimation of the MW of identified recombinant proteins. After electrophoresis, protein is detected generally by staining with Coomassie blue and the stained gel is scanned using a densitometer to determine the percentage of protein present in each band. In this manner, the percentage of protein present in the band corresponding to the recombinant protein of interest may be determined. It is not necessary that Coomassie blue be employed for the detection of protein, a number of fluorescent dyes [e.g., Sypro orange S-6651 (Molecular Probes, Eugene, OR)] may be employed and the stained gel scanned using a fluorimager [e.g., Fluor Imager SI (Molecular Dynamics, Sunnyvale, CA)].

"A host cell capable of expressing a recombinant protein as a soluble protein at a level greater than or equal to 0.25% of the total soluble cellular protein" is a host cell in which the amount of soluble recombinant protein present represents at least 0.25% of the total cellular protein. As used herein "total soluble cellular protein" refers to a clarified PEI lysate prepared as described in Example 31(c)(iv). Briefly, cells are harvested following induction of expression of recombinant protein (at a point of maximal expression). The cells are resuspended in cell resuspension buffer (CRB: 50 mM NaPO<sub>4</sub>, 0.5 M NaCl, 40 mM imidazole, pH 6.8) to create a 20% cell suspension (wet weight of cells/volume of CRB) and cell lysates are prepared as described in Example 31(c)(iv) (*i.e.*, sonication or homogenization followed by centrifugation). The cell lysate is then flocculated utilizing polyethyleneimine (PEI) prior to centrifugation. PEI (a 2% solution in dH<sub>2</sub>O, pH 7.5 with HCl) is added to the cell lysate to a final concentration of 0.2%, and stirred for 20 min at room temperature prior to centrifugation [8,500 rpm in JA10 rotor (Beckman) for 30 minutes at 4°C]. This treatment removes RNA, DNA and cell wall components, resulting in a clarified, low viscosity lysate ("PEI clarified lysate"). The recombinant protein present in the PEI clarified lysate is then

purified (e.g., by chromatography on an IDA column for his-tagged proteins). The amount of purified recombinant protein (i.e., the eluted protein) is divided by the concentration of protein present in the PEI clarified lysate (typically 8 mg/ml when using a 20% cell suspension as the starting material) and multiplied by 100 to determine what percentage of total soluble cellular protein is comprised of the soluble recombinant protein (see Example 33b).

As used herein, the term "fusion protein" refers to a chimeric protein containing the protein of interest (i.e., *C. botulinum* toxin A, B, C, D, E, F, or G and fragments thereof) joined to an exogenous protein fragment (the fusion partner which consists of a non-toxin protein). The fusion partner may enhance solubility of the *C. botulinum* protein as expressed in a host cell, may provide an affinity tag to allow purification of the recombinant fusion protein from the host cell or culture supernatant, or both. If desired, the fusion protein may be removed from the protein of interest (i.e., toxin protein or fragments thereof) prior to immunization by a variety of enzymatic or chemical means known to the art.

As used herein the term "non-toxin protein" or "non-toxin protein sequence" refers to that portion of a fusion protein which comprises a protein or protein sequence which is not derived from a bacterial toxin protein.

The term "protein of interest" as used herein refers to the protein whose expression is desired within the fusion protein. In a fusion protein the protein of interest will be joined or fused with another protein or protein domain, the fusion partner, to allow for enhanced stability of the protein of interest and/or ease of purification of the fusion protein.

As used herein, the term "maltose binding protein" refers to the maltose binding protein of *E. coli*. A portion of the maltose binding protein may be added to a protein of interest to generate a fusion protein; a portion of the maltose binding protein may merely enhance the solubility of the resulting fusion protein when expressed in a bacterial host. On the other hand, a portion of the maltose binding protein may allow affinity purification of the fusion protein on an amylose resin.

As used herein, the term "poly-histidine tract" when used in reference to a fusion protein refers to the presence of two to ten histidine residues at either the amino- or carboxy-terminus of a protein of interest. A poly-histidine tract of six to ten residues is preferred. The poly-histidine tract is also defined functionally as being a number of consecutive histidine

residues added to the protein of interest which allows the affinity purification of the resulting fusion protein on a nickel-chelate or IDA column.

As used herein, the term "purified" or "to purify" refers to the removal of contaminants from a sample. For example, antitoxins are purified by removal of  
5 contaminating non-immunoglobulin proteins; they are also purified by the removal of immunoglobulin that does not bind toxin. The removal of non-immunoglobulin proteins and/or the removal of immunoglobulins that do not bind toxin results in an increase in the percent of toxin-reactive immunoglobulins in the sample. In another example, recombinant toxin polypeptides are expressed in bacterial host cells and the toxin polypeptides are purified  
10 by the removal of host cell proteins; the percent of recombinant toxin polypeptides is thereby increased in the sample. Additionally, the recombinant toxin polypeptides are purified by the removal of host cell components such as lipopolysaccharide (*e.g.*, endotoxin).

The term "recombinant DNA molecule" as used herein refers to a DNA molecule which is comprised of segments of DNA joined together by means of molecular biological  
15 techniques.

The term "recombinant protein" or "recombinant polypeptide" as used herein refers to a protein molecule which is expressed from a recombinant DNA molecule.

The term "native protein" as used herein refers to a protein which is isolated from a natural source as opposed to the production of a protein by recombinant means.

As used herein the term "portion" when in reference to a protein (as in "a portion of a  
20 given protein") refers to fragments of that protein. The fragments may range in size from four amino acid residues to the entire amino acid sequence minus one amino acid.

As used herein "soluble" when in reference to a protein produced by recombinant DNA technology in a host cell is a protein which exists in solution in the cytoplasm of the  
25 host cell; if the protein contains a signal sequence the soluble protein is exported to the periplasmic space in bacteria hosts and is secreted into the culture medium in eucaryotic cells capable of secretion or by bacterial host possessing the appropriate genes (*i.e.*, the *kil* gene). In contrast, an insoluble protein is one which exists in denatured form inside cytoplasmic granules (called inclusion bodies) in the host cell. High level expression (*i.e.*, greater than 10-  
30 20 mg recombinant protein/liter of bacterial culture) of recombinant proteins often results in the expressed protein being found in inclusion bodies in the bacterial host cells. A soluble

protein is a protein which is not found in an inclusion body inside the host cell or is found both in the cytoplasm and in inclusion bodies and in this case the protein may be present at high or low levels in the cytoplasm.

5 A distinction is drawn between a soluble protein (*i.e.*, a protein which when expressed in a host cell is produced in a soluble form) and a "solubilized" protein. An insoluble recombinant protein found inside an inclusion body may be solubilized (*i.e.*, rendered into a soluble form) by treating purified inclusion bodies with denaturants such as guanidine hydrochloride, urea or sodium dodecyl sulfate (SDS). These denaturants must then be removed from the solubilized protein preparation to allow the recovered protein to renature (refold). Not all proteins will refold into an active conformation after solubilization in a denaturant and removal of the denaturant. Many proteins precipitate upon removal of the denaturant. SDS may be used to solubilize inclusion bodies and will maintain the proteins in solution at low concentration. However, dialysis will not always remove all of the SDS (SDS can form micelles which do not dialyze out); therefore, SDS-solubilized inclusion body protein is soluble but not refolded.

10 A distinction is drawn between proteins which are soluble (*i.e.*, dissolved) in a solution devoid of significant amounts of ionic detergents (*e.g.*, SDS) or denaturants (*e.g.*, urea, guanidine hydrochloride) and proteins which exist as a suspension of insoluble protein molecules dispersed within the solution. A soluble protein will not be removed from a solution containing the protein by centrifugation using conditions sufficient to remove bacteria present in a liquid medium (*i.e.*, centrifugation at 12,000 x g for 4-5 minutes). For example, to test whether two proteins, protein A and protein B, are soluble in solution, the two proteins are placed into a solution selected from the group consisting of PBS-NaCl (PBS containing 0.5 M NaCl), PBS-NaCl containing 0.2% Tween 20, PBS, PBS containing 0.2% Tween 20, PBS-C (PBS containing 2 mM CaCl<sub>2</sub>), PBS-C containing either 0.1 or 0.5 % Tween 20, PBS-C containing either 0.1 or 0.5% NP-40, PBS-C containing either 0.1 or 0.5% Triton X-100, PBS-C containing 0.1% sodium deoxycholate. The mixture containing proteins A and B is then centrifuged at 5000 x g for 5 minutes. The supernatant and pellet formed by centrifugation are then assayed for the presence of protein A and B. If protein A is found in the supernatant and not in the pellet [except for minor amounts (*i.e.*, less than 10%) as a result of trapping], protein is said to be soluble in the solution tested. If the majority of

protein B is found in the pellet (*i.e.*, greater than 90%), then protein B is said to exist as a suspension in the solution tested.

As used herein, the term "therapeutic amount" refers to that amount of antitoxin required to neutralize the pathologic effects of one or more clostridial toxins in a subject.

5 The term "pyrogen" as used herein refers to a fever-producing substance. Pyrogens may be endogenous to the host (*e.g.*, prostaglandins) or may be exogenous compounds (*e.g.*, bacterial endo- and exotoxins, nonbacterial compounds such as antigens and certain steroid compounds, etc.). The presence of pyrogen in a pharmaceutical solution may be detected using the U.S. Pharmacopeia (USP) rabbit fever test (United States Pharmacopeia, Vol. XXII  
10 (1990) United States Pharmacopoeial Convention, Rockville, MD, p. 151).

The term "endotoxin" as used herein refers to the high molecular weight complexes associated with the outer membrane of gram-negative bacteria. Unpurified endotoxin contains lipids, proteins and carbohydrates. Highly purified endotoxin does not contain protein and is referred to as lipopolysaccharide (LPS). Because unpurified endotoxin is of concern in the  
15 production of pharmaceutical compounds (*e.g.*, proteins produced in *E. coli* using recombinant DNA technology), the term endotoxin as used herein refers to unpurified endotoxin. Bacterial endotoxin is a well known pyrogen.

As used herein, the term "endotoxin-free" when used in reference to a composition to be administered parenterally (with the exception of intrathecal administration) to a host means  
20 that the dose to be delivered contains less than 5 EU/kg body weight [FDA Guidelines for Parenteral Drugs (December 1987)]. Assuming a weight of 70 kg for an adult human, the dose must contain less than 350 EU to meet FDA Guidelines for parenteral administration. Endotoxin levels are measured herein using the Limulus Amebocyte Lysate (LAL) test (Limulus Amebocyte Lysate Pyrochrome<sup>TM</sup>, Associates of Cape Cod, Inc. Woods Hole, MA).  
25 To measure endotoxin levels in preparations of recombinant proteins, 0.5 ml of a solution comprising 0.5 mg of purified recombinant protein in 50 mM NaPO<sub>4</sub>, pH 7.0, 0.3M NaCl and 10% glycerol is used in the LAL assay according to the manufacturer's instructions for the endpoint chromogenic without diazo-coupling method [the specific components of the buffer containing recombinant protein to be analyzed in the LAL test are not important; any buffer  
30 having a neutral pH may be employed (see for example, alternative buffers employed in Examples 34, 40 and 45)]. Compositions containing less than or equal to than 250 endotoxin

units (EU)/mg of purified recombinant protein are herein defined as "substantially endotoxin-free." Preferably the composition contains less than or equal to 100, and most preferably less than or equal to 60, (EU)/mg of purified recombinant protein. Typically, administration of bacterial toxins or toxoids to adult humans for the purpose of vaccination involves doses of about 10-500 µg protein/dose. Therefore, administration of 10-500 µg of a purified recombinant protein to a 70 kg human, wherein said purified recombinant protein preparation contains 60 EU/mg protein, results in the introduction of only 0.6 to 30 EU (*i.e.*, 0.2 to 8.6% of the maximum allowable endotoxin burden per parenteral dose). Administration of 10-500 µg of a purified recombinant protein to a 70 kg human, wherein said purified recombinant protein preparation contains 250 EU/mg protein, results in the introduction of only 2.5 to 125 EU (*i.e.*, 0.7 to 36% of the maximum allowable endotoxin burden per parenteral dose).

The LAL test is accepted by the U.S. FDA as a means of detecting bacterial endotoxins (21 C.F.R. §§ 660.100 -105). Studies have shown that the LAL test is equivalent or superior to the USP rabbit pyrogen test for the detection of endotoxin and thus the LAL test can be used as a surrogate for pyrogenicity studies in animals [F.C. Perason, *Pyrogens: endotoxins, LAL testing and depyrogenation*, Marcel Dekker, New York (1985), pp.150-155]. The FDA Bureau of Biologics accepts the LAL assay in place of the USP rabbit pyrogen test so long as the LAL assay utilized is shown to be as sensitive as, or more sensitive as the rabbit test [Fed. Reg., 38, 26130 (1980)].

The term "monovalent" when used in reference to a clostridial vaccine refers to a vaccine which is capable of provoking an immune response in a host animal directed against a single type of clostridial toxin. For example, if immunization of a host with *C. botulinum* type A toxin vaccine induces antibodies in the immunized host which protect against a challenge with type A toxin but not against challenge with type B, C, D, E, F or G toxins, then the type A vaccine is said to be monovalent. In contrast, a "multivalent" vaccine provokes an immune response in a host animal directed against several (*i.e.*, more than one) clostridial toxins. For example, if immunization of a host with a vaccine comprising *C. botulinum* type A and B toxins induces the production of antibodies which protect the host against a challenge with both type A and B toxin, the vaccine is said to be multivalent (in particular, this hypothetical vaccine is bivalent).



As used herein the term "immunogenically-effective amount" refers to that amount of an immunogen required to invoke the production of protective levels of antibodies in a host upon vaccination.

The term "protective level", when used in reference to the level of antibodies induced upon immunization of the host with an immunogen which comprises a bacterial toxin, means a level of circulating antibodies sufficient to protect the host from challenge with a lethal dose of the toxin.

As used herein the terms "protein" and "polypeptide" refer to compounds comprising amino acids joined via peptide bonds and are used interchangeably.

The terms "toxin" and "neurotoxin" when used in reference to toxins produced by members (*i.e.*, species and strains) of the genus *Clostridium* are used interchangeably and refer to the proteins which are poisonous to nerve tissue.

The term "receptor-binding domain" when used in reference to a *C. botulinum* toxin refers to the carboxy-terminal portion of the heavy chain (H<sub>C</sub> or the C' fragment) of the toxin which is presumed to be responsible for the binding of the active toxin (*i.e.*, the derivative toxin comprising the H and L chains joined via disulfide bonds) to receptors on the surface of synaptosomes. The receptor-binding domain for *C. botulinum* type A toxin is defined herein as comprising amino acid residues 861 through 1296 of SEQ ID NO:28. The receptor-binding domain for *C. botulinum* type B toxin is defined herein as comprising amino acid residues 848 through 1291 of SEQ ID NO:40 (strain Eklund 17B). The receptor-binding domain of *C. botulinum* type C1 toxin is defined herein as comprising amino acid residues 856 through 1291 of SEQ ID NO:60. The receptor-binding domain of *C. botulinum* type D toxin is defined herein as comprising amino acid residues 852 through 1276 of SEQ ID NO:66. The receptor-binding domain of *C. botulinum* type E toxin is defined herein as comprising amino acid residues 835 through 1250 of SEQ ID NO:50 (Beluga strain). The receptor-binding domain of *C. botulinum* type F toxin is defined herein as comprising amino acid residues 853 through 1274 of SEQ ID NO:71. The receptor-binding domain of *C. botulinum* type G toxin is defined herein as comprising amino acid residues 853 through 1297 of SEQ ID NO:77. Within a given serotype, small variations in the primary amino acid sequence of the botulinical toxins isolated from different strains has been reported [Whelan *et*

5 *al.* (1992), *supra* and Minton (1995) Curr. Top. Microbiol. Immunol. 195:161-194]. The present invention contemplates fusion proteins comprising the receptor-binding domain of *C. botulinum* toxins from serotypes A-G including the variants found among different strains within a given serotype. The receptor-binding domains listed above are used as the prototype for each strain within a serotype. Fusion proteins containing an analogous region from a strain other than the prototype strain are encompassed by the present invention.

10 Fusion proteins comprising the receptor binding domain (*i.e.*, C' fragment) of botulinum toxins may include amino acid residues located beyond the termini of the domains defined above. For example, the pHisBotB protein contains amino acid residues 846-1291 of SEQ ID NO:40; this fusion protein thus comprises the receptor-binding domain for *C. botulinum* type B toxin as defined above (*i.e.*, Ile-848 through Glu-1291). Similarly, pHisBotE contains amino acid residues 827-1252 of SEQ ID NO:50 and pHisBotG contains amino acid residues 851-1297 of SEQ ID NO:77. Thus, both pHisBotE and pHisBotG fusion proteins contain a few amino acids located beyond the N-terminus of the defined receptor-binding domain.

15 The terms "native gene" or "native gene sequences" are used to indicate DNA sequences encoding a particular gene which contain the same DNA sequences as found in the gene as isolated from nature. In contrast, "synthetic gene sequences" are DNA sequences which are used to replace the naturally occurring DNA sequences when the naturally occurring sequences cause expression problems in a given host cell. For example, naturally-occurring DNA sequences encoding codons which are rarely used in a host cell may be replaced (*e.g.*, by site-directed mutagenesis) such that the synthetic DNA sequence represents a more frequently used codon. The native DNA sequence and the synthetic DNA sequence will preferably encode the same amino acid sequence.

## 25 SUMMARY OF THE INVENTION

30 The present invention relates to the production of polypeptides derived from toxins particularly in recombinant host cells. In one embodiment, the present invention provides a host cell containing a recombinant expression vector, said vector encoding a protein comprising at least a portion of a *Clostridium botulinum* toxin, said toxin selected from the group consisting of type B toxin and type E toxin. The present invention is not limited by the nature of sequences encoding portions of the *C. botulinum* toxin. These sequences may be

derived from the native gene sequences or alternatively they may comprise synthetic gene sequences. Synthetic gene sequences are employed when expression of the native gene sequences is problematic in a given host cell (e.g., when the native gene sequences contain sequences resembling yeast transcription termination signals and the desired host cell is a yeast cell).

In one embodiment, the host cell is capable of expressing the recombinant *C. botulinum* toxin protein at a level greater than or equal to 2% to 40% of the total cellular protein and preferably at a level greater than or equal to 5% of the total cellular protein. In another embodiment, the host cell is capable of expressing the recombinant *C. botulinum* toxin protein as a soluble protein at a level greater than or equal to 0.25% of the total cellular protein and preferably at a level greater than or equal to 0.25% to 10% of the total cellular protein.

The present invention is not limited by the nature of the host cell employed for the production of recombinant *C. botulinum* toxin proteins. In a preferred embodiment, the host cell is an *E. coli* cell. In another preferred embodiment, the host cell is an insect cell; particularly preferred insect host cells are *Spodoptera frugiperda* (Sf9) cells. In another preferred embodiment, the host cell is a yeast cell; particularly preferred yeast cells are *Pichia pastoris* cells.

In another embodiment, the invention provides a host cell containing a recombinant expression vector, said vector encoding a fusion protein comprising a non-toxin protein sequence and at least a portion of a *Clostridium botulinum* toxin, said toxin selected from the group consisting of type B toxin and type E toxin. The invention is not limited by the nature of the portion of the *Clostridium botulinum* toxin selected. In a preferred embodiment, the portion of the toxin comprises the receptor binding domain (i.e., the C fragment of the toxin).

The present invention is not limited by the nature of the non-toxin protein sequence employed. In a preferred embodiment, the non-toxin protein sequence comprises a poly-histidine tract. A number of alternative fusion tags or fusion partners are known to the art (e.g., MBP, GST, protein A, etc.) and may be employed for the production of fusion proteins comprising a portion of a botulin toxin.

The present invention further provides a vaccine comprising a fusion protein, said fusion protein comprising a non-toxin protein sequence and at least a portion of a *Clostridium botulinum* toxin, said toxin selected from the group consisting of type B toxin and type E toxin. The vaccine may be a monovalent vaccine (*i.e.*, containing only a toxin B fusion protein or a toxin E fusion protein), a bivalent vaccine (*i.e.*, containing both a toxin B fusion protein and a toxin E fusion protein) or a trivalent or higher valency vaccine. In a preferred embodiment, the toxin B fusion protein and/or toxin E fusion protein is combined with a fusion protein comprising a non-toxin protein sequence and at least a portion of *Clostridium botulinum* type A toxin. The present invention is not limited by the nature of the portion of the *Clostridium botulinum* toxin selected. In a preferred embodiment, the portion of the toxin comprises the receptor binding domain (*i.e.*, the C fragment of the toxin). The present invention is not limited by the nature of the non-toxin protein sequence employed. In a preferred embodiment, the non-toxin protein sequence comprises a poly-histidine tract. A number of alternative fusion tags or fusion partners are known to the art (*e.g.*, MBP, GST, protein A, etc.) and may be employed for the generation of fusion proteins comprising vaccines. When a fusion partner (*i.e.*, the non-toxin protein sequence) is employed for the production of a recombinant *C. botulinum* toxin protein, the fusion partner may be removed from the recombinant *C. botulinum* toxin protein if desired (*i.e.*, prior to administration of the protein to a subject) using a variety of methods known to the art (*e.g.*, digestion of fusion proteins containing FactorXa or thrombin recognition sites with the appropriate enzyme). A number of the pETHis vectors employed herein provide an N-terminal his-tag followed by a FactorXa cleavage site (see Example 28a); the botulinum C fragment sequences follow the FactorXa site and thus, FactorXa can be used to remove the his-tag from the botulinum fusion protein. In a preferred embodiment, the vaccine is substantially endotoxin-free.

The present invention is not limited by the method employed for the generation of vaccine comprising fusion proteins comprising a non-toxin protein sequence and at least a portion of a *Clostridium botulinum* toxin. The fusion proteins may be produced by recombinant DNA means using either native or synthetic gene sequences expressed in a host cell. The present invention is not limited to the production of vaccines using recombinant host cells; cell free *in vitro* transcription/translation systems may be employed for the

expression of the nucleic acid constructs encoding the fusion proteins of the present invention. An example of such a cell-free system is the commercially available TnT™ Coupled Reticulocyte Lysate System (Promega Corporation, Madison, WI). Alternatively, the fusion proteins of the present invention may be generated by synthetic means (*i.e.*, peptide synthesis).

The present invention further provides a method of generating antibody directed against a *Clostridium botulinum* toxin comprising: a) providing in any order: i) an antigen comprising a fusion protein comprising a non-toxin protein sequence and at least a portion of a *Clostridium botulinum* toxin, said toxin selected from the group consisting of type B toxin and type E toxin, and ii) a host; and b) immunizing the host with the antigen so as to generate an antibody. In a preferred embodiment, the antigen used to immunize the host also contains a fusion protein comprising a non-toxin protein sequence and at least a portion of *Clostridium botulinum* type A toxin. The present invention is not limited by the nature of the portion of the *Clostridium botulinum* toxin selected. In a preferred embodiment, the portion of the toxin comprises the receptor binding domain (*i.e.*, the C fragment of the toxin). The present invention is not limited by the nature of the non-toxin protein sequence employed. In a preferred embodiment, the non-toxin protein sequence comprises a poly-histidine tract. A number of alternative fusion tags or fusion partners are known to the art (*e.g.*, MBP, GST, protein A, etc.) and may be employed for the generation of fusion proteins comprising vaccines. When a fusion partner (*i.e.*, the non-toxin protein sequence) is employed for the production of a recombinant *C. botulinum* toxin protein, the fusion partner may be removed from the recombinant *C. botulinum* toxin protein if desired (*i.e.*, prior to administration of the protein to a subject) using a variety of methods known to the art (*e.g.*, digestion of fusion proteins containing FactorXa or thrombin recognition sites with the appropriate enzyme).

The present invention is not limited by the nature of the host employed for the production of the antibodies of the invention. In a preferred embodiment, the host is a mammal, preferably a human. The antibodies of the present invention may be generated using non-mammalian hosts such as birds, preferably chickens. In a preferred embodiment the method of the present invention further comprised the step c) of collecting the antibodies

from the host. In yet another embodiment, the method of the present invention further comprises the step d) of purifying the antibodies.

The present invention further provides antibodies raised according to the above methods.

5 The present invention further contemplates multivalent vaccines comprising at least two recombinant *C. botulinum* toxin proteins derived from the group consisting of *C. botulinum* serotypes A, B, C, D, E, F, and G. The invention contemplates bivalent, trivalent, quadravalent, pentavalent, heptavalent and septivalent vaccines comprising recombinant *C. botulinum* toxin proteins. Preferably the recombinant *C. botulinum* toxin protein comprises  
10 the receptor binding domain (i.e., C fragment) of the toxin.

## DESCRIPTION OF THE INVENTION

The present invention contemplates vaccinating humans and other animals with polypeptides derived from *C. botulinum* neurotoxins which are substantially endotoxin-free.  
15 These botulinal peptides are also useful for the production of antitoxin. Anti-botulinal toxin antitoxin is useful for the treatment of patients effected by or at risk of symptoms due to the action of *C. botulinum* toxins. The organisms, toxins and individual steps of the present invention are described separately below.

### 20 I. *Clostridium* Species, Clostridial Diseases And Associated Toxins

A preferred embodiment of the method of the present invention is directed toward obtaining antibodies against *Clostridium* species, their toxins, enzymes or other metabolic by-products, cell wall components, or synthetic or recombinant versions of any of these compounds. It is contemplated that these antibodies will be produced by immunization of  
25 humans or other animals. It is not intended that the present invention be limited to any particular toxin or any species of organism. In one embodiment, toxins from all *Clostridium* species are contemplated as immunogens. Examples of these toxins include the neuraminidase toxin of *C. butyricum*, *C. sordellii* toxins HT and LT, toxins A, B, C, D, E, F, and G of *C. botulinum* and the numerous *C. perfringens* toxins. In one preferred embodiment, toxins A,

B. and E. of *C. botulinum* are contemplated as immunogens. Table 2 above lists various *Clostridium* species, their toxins and some antigens associated with disease.

TABLE 2

## Clostridial Toxins

Organism	Toxins and Disease-Associated Antigens
<i>C. botulinum</i>	A, B, C <sub>1</sub> , C <sub>2</sub> , D, E, F, G
<i>C. butyricum</i>	Neuraminidase
<i>C. difficile</i>	A, B, Enterotoxin (not A nor B), Motility Altering Factor, Low Molecular Weight Toxin, Others
<i>C. perfringens</i>	$\alpha$ , $\beta$ , $\epsilon$ , $\iota$ , $\gamma$ , $\delta$ , $\nu$ , $\theta$ , $\kappa$ , $\lambda$ , $\mu$ , $\upsilon$
<i>C. sordelli</i> <i>C. bifermentans</i>	HT, LT, $\alpha$ , $\beta$ , $\gamma$
<i>C. novyi</i>	$\alpha$ , $\beta$ , $\gamma$ , $\delta$ , $\epsilon$ , $\zeta$ , $\nu$ , $\theta$
<i>C. septicum</i>	$\alpha$ , $\beta$ , $\gamma$ , $\delta$
<i>C. histolyticum</i>	$\alpha$ , $\beta$ , $\gamma$ , $\delta$ , $\epsilon$ plus additional enzymes
<i>C. chauvoei</i>	$\alpha$ , $\beta$ , $\gamma$ , $\delta$

It is not intended that antibodies produced against one toxin will only be used against that toxin. It is contemplated that antibodies directed against one toxin (e.g., *C. perfringens* type A enterotoxin) may be used as an effective therapeutic against one or more toxin(s) produced by other members of the genus *Clostridium* or other toxin producing organisms (e.g., *Bacillus cereus*, *Staphylococcus aureus*, *Streptococcus mutans*, *Acinetobacter calcoaceticus*, *Pseudomonas aeruginosa*, other *Pseudomonas* species, etc.). It is further contemplated that antibodies directed against the portion of the toxin which binds to mammalian membranes (e.g., *C. perfringens* enterotoxin A) can also be used against other organisms. It is contemplated that these membrane binding domains are produced synthetically and used as immunogens.

## II. Obtaining Antibodies In Non-Mammals

A preferred embodiment of the method of the present invention for obtaining antibodies involves immunization. However, it is also contemplated that antibodies could be obtained from non-mammals without immunization. In the case where no immunization is

contemplated, the present invention may use non-mammals with preexisting antibodies to toxins as well as non-mammals that have antibodies to whole organisms by virtue of reactions with the administered antigen. An example of the latter involves immunization with synthetic peptides or recombinant proteins sharing epitopes with whole organism components.

5 In a preferred embodiment, the method of the present invention contemplates immunizing non-mammals with bacterial toxin(s). It is not intended that the present invention be limited to any particular toxin. In one embodiment, toxin from all clostridial bacteria sources (see Table 2) are contemplated as immunogens. Examples of these toxins are *C. butyricum* neuraminidase toxin, toxins A, B, C, D, E, F, and G from *C. botulinum*,  
10 *C. perfringens* toxins  $\alpha$ ,  $\beta$ ,  $\epsilon$ , and  $\iota$ , and *C. sordellii* toxins HT and LT. In a preferred embodiment, *C. botulinum* toxins A, B, C, D, E, and F (or fragments thereof) are contemplated as immunogens.

A particularly preferred embodiment involves the use of bacterial toxin protein or fragments of toxin proteins produced by molecular biological means (i.e., recombinant toxin  
15 proteins). In a preferred embodiment, the immunogen comprises the receptor-binding domain (i.e., the ~50 kD carboxy-terminal portion of the heavy chain; also referred to as the C' fragment) of *C. botulinum* serotype A neurotoxin produced by recombinant DNA technology. In another preferred embodiment, the immunogen comprises the receptor-binding domain of *C. botulinum* serotype B neurotoxin produced by recombinant DNA technology. In yet  
20 another preferred embodiment, the immunogen comprises the receptor-binding domain region of *C. botulinum* serotype E neurotoxin produced by recombinant DNA technology. In yet another preferred embodiment, the immunogen comprises the receptor-binding domain region of *C. botulinum* serotype C1 neurotoxin produced by recombinant DNA technology. In yet  
25 another preferred embodiment, the immunogen comprises the receptor-binding domain region of *C. botulinum* serotype C2 neurotoxin produced by recombinant DNA technology. In yet another preferred embodiment, the immunogen comprises the receptor-binding domain region of *C. botulinum* serotype D neurotoxin produced by recombinant DNA technology. In yet  
30 another preferred embodiment, the immunogen comprises the receptor-binding domain region of *C. botulinum* serotype F neurotoxin produced by recombinant DNA technology. In a preferred embodiment, the recombinant botulinum toxin proteins are expressed as fusion proteins (e.g., as histidine-tagged proteins). In a still further preferred embodiment, the



immunogen is a multivalent vaccine comprising the receptor-binding domain region of *C. botulinum* toxin from two or more toxins selected from the group consisting of type A, type B, type C (including C1 and C2), type D, type E, and type F toxin.

When immunization is used, the preferred non-mammal is from the class *Aves*. All  
5 birds are contemplated (e.g., duck, ostrich, emu, turkey, etc.). A preferred bird is a chicken. Importantly, chicken antibody does not fix mammalian complement. [See H.N. Benson *et al.*, *J. Immunol.* 87:616 (1961).] Thus, chicken antibody will normally not cause a complement-dependent reaction. [A.A. Benedict and K. Yamaga, "Immunoglobulins and Antibody  
Production in Avian Species," in *Comparative Immunology* (J.J. Marchalonis, ed.), pp. 335-  
10 375, Blackwell, Oxford (1966).] Thus, the preferred antitoxins of the present invention will not exhibit complement-related side effects observed with antitoxins known presently.

When birds are used, it is contemplated that the antibody will be obtained from either the bird serum or the egg. A preferred embodiment involves collection of the antibody from the egg. Laying hens transport immunoglobulin to the egg yolk ("IgY") in concentrations  
15 equal to or exceeding that found in serum. [See R. Patterson *et al.*, *J. Immunol.* 89:272 (1962); and S.B. Carroll and B.D. Stollar, *J. Biol. Chem.* 258:24 (1983).] In addition, the large volume of egg yolk produced vastly exceeds the volume of serum that can be safely obtained from the bird over any given time period. Finally, the antibody from eggs is purer and more homogeneous; there is far less non-immunoglobulin protein (as compared to serum)  
20 and only one class of immunoglobulin is transported to the yolk.

When considering immunization with toxins, one may consider modification of the toxins to reduce the toxicity. In this regard, it is not intended that the present invention be limited by immunization with modified toxin. Unmodified ("native") toxin is also contemplated as an immunogen.

25 It is also not intended that the present invention be limited by the type of modification -- if modification is used. The present invention contemplates all types of toxin modification, including chemical and heat treatment of the toxin. The preferred modification, however, is formaldehyde treatment.

30 It is not intended that the present invention be limited to a particular mode of immunization; the present invention contemplates all modes of immunization, including subcutaneous, intramuscular, intraperitoneal, and intravenous or intravascular injection, as well as *per os* administration of immunogen.

The present invention further contemplates immunization with or without adjuvant. (Adjuvant is defined as a substance known to increase the immune response to other antigens when administered with other antigens.) If adjuvant is used, it is not intended that the present invention be limited to any particular type of adjuvant -- or that the same adjuvant, once used, be used all the time. While the present invention contemplates all types of adjuvant, whether used separately or in combinations, the preferred use of adjuvant is the use of Complete Freund's Adjuvant followed sometime later with Incomplete Freund's Adjuvant. Another preferred use of adjuvant is the use of Gerbu Adjuvant. The invention also contemplates the use of RIBI fowl adjuvant and Quil A adjuvant.

When immunization is used, the present invention contemplates a wide variety of immunization schedules. In one embodiment, a chicken is administered toxin(s) on day zero and subsequently receives toxin(s) in intervals thereafter. It is not intended that the present invention be limited by the particular intervals or doses. Similarly, it is not intended that the present invention be limited to any particular schedule for collecting antibody. The preferred collection time is sometime after day 100.

Where birds are used and collection of antibody is performed by collecting eggs, the eggs may be stored prior to processing for antibody. It is preferred that eggs be stored at 4°C for less than one year.

It is contemplated that chicken antibody produced in this manner can be buffer-extracted and used analytically. While unpurified, this preparation can serve as a reference for activity of the antibody prior to further manipulations (e.g., immunoaffinity purification).

### III. Increasing The Effectiveness Of Antibodies

When purification is used, the present invention contemplates purifying to increase the effectiveness of both non-mammalian antitoxins and mammalian antitoxins. Specifically, the present invention contemplates increasing the percent of toxin-reactive immunoglobulin. The preferred purification approach for avian antibody is polyethylene glycol (PEG) separation.

The present invention contemplates that avian antibody be initially purified using simple, inexpensive procedures. In one embodiment, chicken antibody from eggs is purified by extraction and precipitation with PEG. PEG purification exploits the differential solubility of lipids (which are abundant in egg yolks) and yolk proteins in high concentrations of PEG 8000. [Polson *et al.*, Immunol. Comm. 9:495 (1980).] The technique is rapid, simple, and relatively inexpensive and yields an immunoglobulin fraction that is significantly purer in

terms of contaminating non-immunoglobulin proteins than the comparable ammonium sulfate fractions of mammalian sera and horse antibodies. The majority of the PEG is removed from the precipitated chicken immunoglobulin by treatment with ethanol. Indeed, PEG-purified antibody is sufficiently pure that the present invention contemplates the use of PEG-purified antitoxins in the passive immunization of intoxicated humans and animals.

#### IV. Treatment

The present invention contemplates antitoxin therapy for humans and other animals intoxicated by bacterial toxins. A preferred method of treatment is by intravenous administration of anti-boutlinal antitoxin; oral administration is also contemplated for other clostridial antitoxins.

##### A. Dosage Of Antitoxin

It was noted by way of background that a balance must be struck when administering currently available antitoxin which is usually produced in large animals such as horses: sufficient antitoxin must be administered to neutralize the toxin, but not so much antitoxin as to increase the risk of untoward side effects. These side effects are caused by: i) patient sensitivity to foreign (*e.g.* horse) proteins; ii) anaphylactic or immunogenic properties of non-immunoglobulin proteins; iii) the complement fixing properties of mammalian antibodies; and/or iv) the overall burden of foreign protein administered. It is extremely difficult to strike this balance when, as noted above, the degree of intoxication (and hence the level of antitoxin therapy needed) can only be approximated.

The present invention contemplates significantly reducing side effects so that this balance is more easily achieved. Treatment according to the present invention contemplates reducing side effects by using PEG-purified antitoxin from birds.

In one embodiment, the treatment of the present invention contemplates the use of PEG-purified antitoxin from birds. The use of yolk-derived, PEG-purified antibody as antitoxin allows for the administration of: 1) non(mammalian)-complement-fixing, avian antibody; 2) a less heterogeneous mixture of non-immunoglobulin proteins; and 3) less total protein to deliver the equivalent weight of active antibody present in currently available antitoxins. The non-mammalian source of the antitoxin makes it useful for treating patients who are sensitive to horse or other mammalian sera.

## B. Delivery Of Antitoxin

Although it is not intended to limit the route of delivery, the present invention contemplates a method for antitoxin treatment of bacterial intoxication in which delivery of antitoxin is oral. In one embodiment, antitoxin is delivered in a solid form (e.g., tablets). In an alternative embodiment antitoxin is delivered in an aqueous solution. When an aqueous solution is used, the solution has sufficient ionic strength to solubilize antibody protein, yet is made palatable for oral administration. The delivery solution may also be buffered (e.g., carbonate buffer pH 9.5) which can neutralize stomach acids and stabilize the antibodies when the antibodies are administered orally. In one embodiment the delivery solution is an aqueous solution. In another embodiment the delivery solution is a nutritional formula. Preferably, the delivery solution is infant formula. Yet another embodiment contemplates the delivery of lyophilized antibody encapsulated or microencapsulated inside acid-resistant compounds.

Methods of applying enteric coatings to pharmaceutical compounds are well known to the art [companies specializing in the coating of pharmaceutical compounds are available; for example, The Coating Place (Verona, WI) and AAI (Wilmington, NC)]. Enteric coatings which are resistant to gastric fluid and whose release (*i.e.*, dissolution of the coating to release the pharmaceutical compound) is pH dependent are commercially available [for example, the polymethacrylates Eudragit® L and Eudragit® S (Röhm GmbH)]. Eudragit® S is soluble in intestinal fluid from pH 7.0; this coating can be used to microencapsulate lyophilized antitoxin antibodies and the particles are suspended in a solution having a pH above or below pH 7.0 for oral administration. The microparticles will remain intact and undissolved until they reached the intestines where the intestinal pH would cause them to dissolve thereby releasing the antitoxin.

The invention contemplates a method of treatment which can be administered for treatment of acute intoxication. In one embodiment, antitoxin is administered orally in either a delivery solution or in tablet form, in therapeutic dosage, to a subject intoxicated by the bacterial toxin which served as immunogen for the antitoxin.

The invention also contemplates a method of treatment which can be administered prophylactically. In one embodiment, antitoxin is administered orally, in a delivery solution, in therapeutic dosage, to a subject, to prevent intoxication of the subject by the bacterial toxin which served as immunogen for the production of antitoxin. In another embodiment, antitoxin is administered orally in solid form such as tablets or as microencapsulated particles. Microencapsulation of lyophilized antibody using compounds such as Eudragit® (Rohm

GmbH) or polyethylene glycol, which dissolve at a wide range of pH units, allows the oral administration of solid antitoxin in a liquid form (*i.e.*, a suspension) to recipients unable to tolerate administration of tablets (*e.g.*, children or patients on feeding tubes). In one preferred embodiment the subject is a child. In another embodiment, antibody raised against whole bacterial organism is administered orally to a subject, in a delivery solution, in therapeutic dosage.

#### V. Vaccines Against Clostridial Species

The invention contemplates the generation of mono- and multivalent vaccines for the protection of an animal (particularly humans) against several clostridial species. Of particular interest are vaccines which stimulate the production of a humoral immune response to *C. botulinum*, *C. tetani* and *C. difficile* in humans. The antigens comprising the vaccine preparation may be native or recombinantly produced toxin proteins from the clostridial species listed above. When toxin proteins are used as immunogens they are generally modified to reduce the toxicity. This modification may be by chemical or genetic (*i.e.*, recombinant DNA technology) means. In general genetic detoxification (*i.e.*, the expression of nontoxic fragments in a host cell) is preferred as the expression of nontoxic fragments in a host cell precludes the presence of intact, active toxin in the final preparation. However, when chemical modification is desired, the preferred toxin modification is formaldehyde treatment.

The invention contemplates that recombinant *C. botulinum* toxin proteins be used as antigens in mono- and multivalent vaccine preparations. Soluble, substantially endotoxin-free recombinant *C. botulinum* toxin proteins derived from serotypes A, B and E may be used individually (*i.e.*, as mono-valent vaccines) or in combination (*i.e.*, as a multi-valent vaccine). In addition, the recombinant *C. botulinum* toxin proteins derived from serotypes A, B and E may be used in conjunction with either recombinant or native toxins or toxoids from other serotypes of *C. botulinum*, *C. difficile* and *C. tetani* as antigens for the preparation of these mono- and multivalent vaccines. It is contemplated that, due to the structural similarity of *C. botulinum* and *C. tetani* toxin proteins, a vaccine comprising *C. difficile* and *botulinum* toxin proteins (native or recombinant or a mixture thereof) be used to stimulate an immune response against *C. botulinum*, *C. tetani* and *C. difficile*.

The present invention further contemplates multi-valent vaccines comprising two or more botulinal toxin proteins selected from the group comprising recombinant *C. botulinum* toxin proteins derived from serotypes A, B, C (including C1 and C2), D, E, F and G.

The adverse consequences of exposure to botulinal toxin would be avoided by immunization of subjects at risk of exposure to the toxin with nontoxic preparations which confer immunity such as chemically or genetically detoxified toxin.

Vaccines which confer immunity against one or more of the toxin types A, B, E, F and G would be useful as a means of protecting humans from the deleterious effects of those *C. botulinum* toxins known to affect man. Indeed as the possibility exists that humans could be exposed to any of the seven serotypes of *C. botulinum* toxin (e.g., during biological warfare or the production of toxin in a laboratory setting), multivalent vaccines capable of conferring immunity against toxin types A-G (including both C1 and C2 toxins) would be useful for the protection of humans. Vaccines which confer immunity against one or more of the toxin types C, D and E would be useful for veterinary applications.

The botulinal neurotoxin is synthesized as a single polypeptide chain which is processed into a heavy (H; ~100 kD) and a light (L; ~50 kD) chain by cleavage with proteolytic enzymes; these two chains are held together via disulfide bonds in the active toxin (referred to as derivative toxin) [B.R. DasGupta and H. Sugiyama, Biochem. Biophys. Res. Commun. 48:108 (1972); reviewed in B.R. DasGupta, J. Physiol. 84:220 (1990), H. Sugiyama, Microbiol. Rev. 44:419 (1980) and C.L. Hatheway, Clin. Microbiol. Rev. 3:66 (1990)]. The heavy chain of the active toxin is cleaved by trypsin to produce two fragments termed H<sub>C</sub> (also referred to as H<sub>1</sub> or C) and H<sub>N</sub> (also referred to as H<sub>2</sub> or B). The H<sub>C</sub> fragment (~46 kD) comprises the carboxy end of the H chain. The H<sub>N</sub> fragment (~49 kD) comprises the amino end and remains attached to the L chain (H<sub>N</sub>L). Neither H<sub>C</sub> or H<sub>N</sub>L is toxic. H<sub>C</sub> competes with whole derivative toxin for binding to synaptosomes and therefore H<sub>C</sub> is said to contain the receptor binding site. The H<sub>C</sub> and H<sub>N</sub> fragments of botulinal toxin are analogous to the fragments C and B of tetanus toxin which are produced by papain cleavage. The C fragment of tetanus toxin has been shown to be responsible for the binding of tetanus toxin to purified gangliosides and neuronal cells [Halpern and Loftus, J. Biol. Chem. 268:11188 (1993)].

Antisera raised against purified preparations of isolated botulinal H and L chains have been shown to protect mice against the lethal effects of the toxin; however, the effectiveness of the two antisera differ with the anti-H sera being more potent (H. Sugiyama, *supra*). While the different botulinal toxins show structural similarity to one another, the different

serotypes are reported to be immunologically distinct (*i.e.*, sera raised against one toxin type does not cross-react to a significant degree with other types). Thus, the generation of multivalent vaccines may require the use of more than one type of toxin.

5 *C. botulinum* toxin genes from all seven serotypes have been cloned and sequenced (Minton (1995), *supra*); in addition, partial amino acid sequence is available for a number of *C. botulinum* toxins isolated from different strains within a given serotype. The *C. botulinum* toxins contain about 1250-1300 amino acid residues. On the DNA level, the overall degree of homology between *C. botulinum* serotypes A, B, C, D and E toxins averages between 50 and 60% identity with a greater degree of homology being found between H chain-encoding  
10 regions than between those encoding L chains [Whelan *et al.* (1992) Appl. Environ. Microbiol. 58:2345]. The degree of identity between *C. botulinum* toxins on the amino acid level reflects the level of DNA sequence homology. The most divergent area of DNA and amino acid sequence is found within the carboxy-terminal area of the various *C. botulinum* H chain genes. This portion of the toxin (*i.e.*, H<sub>C</sub> or the C fragment) plays a major role in cell  
15 binding. As toxin from different serotypes is thought to bind to distinct cell receptor molecules, it is not surprising that the toxins diverge significantly over this region.

Within a given serotype, small variations in the primary amino acid sequence of the botulinum toxins isolated from different strains has been reported [Whelan *et al.* (1992), *supra* and Minton (1995), *supra*]. The present invention contemplates fusion proteins comprising  
20 portions of *C. botulinum* toxins from serotypes A-G including the variants found among different strains within a given serotype. The present invention provides oligonucleotide primers which may be used to amplify the C fragment or receptor-binding region of the toxin gene from various strains of *C. botulinum* serotype A, serotype B, serotype C (C1 and C2), serotype D, serotype E, serotype F and serotype G. A large number of different strains of *C.*  
25 *botulinum* serotype A, serotype B, serotype C, serotype D serotype E and serotype F are available from the American Type Culture Collection (ATCC: Rockville, MD). For example, the ATCC provides the following: Type A strains: 174 (ATCC 3502), 457 (ATCC 17862), and NCTC 7272 (ATCC 19397); Type B strains: 34 (ATCC 439), 62A (ATCC 7948), NCA 213 B (ATCC 7949), 13114 (ATCC 8083), 3137 (ATCC 17780), 1347 (ATCC 17841), 2017 (ATCC 17843), 2217 (ATCC 17844), 2254 (ATCC 17845) and VP 1731 (ATCC 25765);  
30 Type C strains: 2220 (ATCC 17782), 2239 (ATCC 17783), 2223 (ATCC 17784; a type C- $\beta$  strain; C- $\beta$  strains produce C2 toxin), 662 (ATCC 17849; a type C- $\alpha$  strain; C- $\alpha$  strains produce mainly C1 toxin and a small amount of C2 toxin), 2021 (ATCC 17850; a type C- $\alpha$

strain) and VPI 3803 (ATCC 25766); Type D strains: ATCC 9633, 2023 (ATCC 17851), and VPI 5995 (ATCC 27517); Type E strains: ATCC 43181, 36208 (ATCC 9564), 2231 (ATCC 17786), 2229 (ATCC 17852), 2279 (ATCC 17854) and 2285 (ATCC 17855) and Type F strains: 202F (ATCC 23387), VPI 4404 (ATCC 25764), VPI 2382 (ATCC 27321) and Langeland (ATCC 35415). Type G strain, 113/30 (NCFB 3012) may be obtained from the National Collection of Food Bacteria (NCFB, AFRC Institute of Food Research, Reading, United Kingdom).

Purification methods have been reported for native toxin types A, B, C, D, E, and F [reviewed in G. Sakaguchi, *Pharmac. Ther.* 19:165 (1983)]. As the different botulin toxins are structurally related, the invention contemplates the expression of any of the botulin toxins (e.g., types A-G) as soluble recombinant fusion proteins.

In particular, methods for purification of the type A botulinum neurotoxin have been developed [L.J. Moberg and H. Sugiyama, *Appl. Environ. Microbiol.* 35:878 (1978)]. Immunization of hens with detoxified purified protein results in the generation of neutralizing antibodies [B.S. Thalley *et al.*, in *Botulinum and Tetanus Neurotoxins*, B.R. DasGupta, ed., Plenum Press, New York (1993), p. 467].

The currently available *C. botulinum* pentavalent vaccine comprising chemically inactivated (i.e., formaldehyde treated) type A, B, C, D and E toxins is not adequate. The efficacy is variable (in particular, only 78% of recipients produce protective levels of anti-type B antibodies following administration of the primary series) and immunization is painful (deep subcutaneous inoculation is required for administration), with adverse reactions being common (moderate to severe local reactions occur in approximately 6% of recipients upon initial injection; this number rises to approximately 11% of individuals who receive booster injections) [Informational Brochure for the Pentavalent (ABCDE) Botulinum Toxoid, Centers for Disease Control]. Preparation of this vaccine is dangerous as active toxin must be handled by laboratory workers.

In general, chemical detoxification of bacterial toxins using agents such as formaldehyde, glutaraldehyde or hydrogen peroxide is not optimal for the generation of vaccines or antitoxins. A delicate balance must be struck between too much and too little chemical modification. If the treatment is insufficient, the vaccine may retain residual toxicity. If the treatment is too excessive, the vaccine may lose potency due to destruction of native immunogenic determinants. Another major limitation of using botulin toxins for the generation of antitoxins or vaccines is the high production expense. For the above reasons,



the development of methods for the production of nontoxic but immunogenic *C. botulinum* toxin proteins is desirable.

The *C. botulinum* and *C. tetanus* toxin proteins have similar structures [reviewed in E.J. Schantz and E.A. Johnson, Microbiol. Rev. 56:80 (1992)]. The carboxy-terminal 50 kD fragment of the tetanus toxin heavy chain (fragment C) is released by papain cleavage and has been shown to be non-toxic and immunogenic. Recombinant tetanus toxin fragment C has been developed as a candidate vaccine antigen [A.J. Makoff *et al.*, Bio/Technology 7:1043 (1989)]. Mice immunized with recombinant tetanus toxin fragment C were protected from challenge with lethal doses of tetanus toxin. No studies have demonstrated that the recombinant tetanus fragment C protein confers immunity against other botulinal toxins such as the *C. botulinum* toxins.

Recombinant tetanus fragment C has been expressed in *E. coli* [A.J. Makoff *et al.*, Bio/Technology, *supra* and Nucleic Acids Res. 17:10191 (1989); J.L. Halpern *et al.*, Infect. Immun. 58:1004 (1990)], yeast [M.A. Romanos *et al.*, Nucleic Acids Res. 19:1461 (1991)] and baculovirus [I.G. Charles *et al.*, Infect. Immun. 59:1627 (1991)]. Synthetic tetanus toxin genes had to be constructed to facilitate expression in yeast (M.A. Romanos *et al.*, *supra*) and *E. coli* [A.J. Makoff *et al.*, Nucleic Acids Res., *supra*], due to the high A-T content of the tetanus toxin gene sequences. High A-T content is a common feature of clostridial genes [M.R. Popoff *et al.*, Infect. Immun. 59:3673 (1991); H.F. LaPenotiere *et al.*, in *Botulinum and Tetanus Neurotoxins*, B.R. DasGupta, ed., Plenum Press, New York (1993), p. 463] which creates expression difficulties in *E. coli* and yeast due primarily to altered codon usage frequency and fortuitous polyadenylation sites, respectively.

The C fragment of the *C. botulinum* type A neurotoxin heavy chain has been evaluated as a vaccine candidate. The *C. botulinum* type A neurotoxin gene has been cloned and sequenced [D.E. Thompson *et al.*, Eur. J. Biochem. 189:73 (1990)]. The C fragment of the type A toxin was expressed as either a fusion protein comprising the botulinal C fragment fused with the maltose binding protein (MBP) or as a native protein [H.F. LaPenotiere *et al.*, (1993) *supra*, H.F. LaPenotiere *et al.*, Toxicon. 33:1383 (1995) and Middlebrook and Brown (1995), Curr. Top. Microbiol. Immunol. 195:89-122]. The plasmid construct encoding the native protein was reported to be unstable, while the fusion protein was expressed primarily in inclusion bodies as insoluble protein. Immunization of mice with crudely purified MBP fusion protein resulted in protection against IP challenge with 3 LD<sub>50</sub> doses of toxin [LaPenotiere *et al.*, (1993) and (1995), *supra*]. However, this recombinant *C. botulinum* type

A toxin C fragment/MBP fusion protein is not a suitable immunogen for the production of vaccines as it is expressed as an insoluble protein in *E. coli*. Furthermore, this recombinant *C. botulinum* type A toxin C fragment/MBP fusion protein was not shown to be substantially free of endotoxin contamination. Experience with recombinant *C. botulinum* type A toxin C fragment/MBP fusion proteins shows that the presence of the MBP on the fusion protein greatly complicates the removal of endotoxin from preparations of the recombinant fusion protein (*see* Ex. 24, *infra*). Expression of a synthetic gene encoding *C. botulinum* type A toxin C fragment as a soluble protein excreted from insect cells has been reported [Middlebrook and Brown (1995), *supra*]; no details regarding the level of expression achieved or the presence of endotoxin or other pyrogens were provided. Like the insoluble protein expressed in *E. coli*, immunization with the recombinant protein produced in insect cells was reported to protect mice from challenge with *C. botulinum* toxin A.

Inclusion body protein must be solubilized prior to purification and/or administration to a host. The harsh treatment of inclusion body protein needed to accomplish this solubilization may reduce the immunogenicity of the purified protein. Ideally, recombinant proteins to be used as vaccines are expressed as soluble proteins at high levels (*i.e.*, greater than or equal to about 0.75% of total cellular protein) in *E. coli* or other host cells (*e.g.*, yeast, insect cells, etc.). This facilitates the production and isolation of sufficient quantities of the immunogen in a highly purified form (*i.e.*, substantially free of endotoxin or other pyrogen contamination). The ability to express recombinant toxin proteins as soluble proteins in *E. coli* is advantageous due to the low cost of growth compared to insect or mammalian tissue culture cells.

The *C. botulinum* type B neurotoxin gene has been cloned and sequenced from two strains of *C. botulinum* type B [Whelan *et al.* (1992) *Appl. Environ. Microbiol.* 58:2345 (Danish strain) and Hutson *et al.* (1994) *Curr. Microbiol.* 28:101 (Eklund 17B strain)]. The nucleotide sequence of the toxin gene derived from the Eklund 17B strain (ATCC 25765) is available from the EMBL/GenBank sequence data banks under the accession number X71343; the nucleotide sequence of the coding region is listed in SEQ ID NO:39. The amino acid sequence of the *C. botulinum* type B neurotoxin derived from the strain Eklund 17B is listed in SEQ ID NO:40. The nucleotide sequence of the *C. botulinum* serotype B toxin gene derived from the Danish strain is listed in SEQ ID NO:41. The amino acid sequence of the *C. botulinum* type B neurotoxin derived from the Danish strain is listed in SEQ ID NO:42.

The *C. botulinum* type B neurotoxin gene is synthesized as a single polypeptide chain which is processed to form a dimer composed of a light and a heavy chain linked via disulfide bonds. The light chain is responsible for pharmacological activity (*i.e.*, inhibition of the release of acetylcholine at the neuromuscular junction). The N-terminal portion of the heavy chain is thought to mediate channel formation while the C-terminal portion mediates toxin binding; the type B neurotoxin has been reported to exist as a mixture of predominantly single chain with some double chain (Whelan *et al.*, *supra*). The 50 kD carboxy-terminal portion of the heavy chain is referred to as the C fragment or the H<sub>C</sub> domain. The present invention reports for the first time, the expression of the C fragment of *C. botulinum* type B toxin in heterologous hosts (*e.g.*, *E. coli*).

The *C. botulinum* type E neurotoxin gene has been cloned and sequenced from a number of different strains [Poulet *et al.* (1992) Biochem. Biophys. Res. Commun. 183:107; Whelan *et al.* (1992) Eur. J. Biochem. 204:657; and Fujii *et al.* (1993) J. Gen. Microbiol. 139:79]. The nucleotide sequence of the type E toxin gene is available from the EMBL sequence data bank under accession numbers X62089 (strain Beluga) and X62683 (strain NCTC 11219); the nucleotide sequence of the coding region (strain Beluga) is listed in SEQ ID NO:45. The amino acid sequence of the *C. botulinum* type E neurotoxin derived from strain Beluga is listed in SEQ ID NO:46. The type E neurotoxin gene is synthesized as a single polypeptide chain which may be converted to a double-chain form (*i.e.*, a heavy chain and a light chain) by cleavage with trypsin; unlike the type A neurotoxin, the type E neurotoxin exists essentially only in the single-chain form. The 50 kD carboxy-terminal portion of the heavy chain is referred to as the C fragment or the H<sub>C</sub> domain. The present invention reports for the first time, the expression of the C fragment of *C. botulinum* type E toxin in heterologous hosts (*e.g.*, *E. coli*).

The *C. botulinum* type C1, D, F and G neurotoxin genes have been cloned and sequenced. The nucleotide and amino acid sequences of these genes and toxins are provided herein. The invention provides methods for the expression of the C fragment from each of these toxin genes in heterologous hosts and the purification of the resulting recombinant proteins.

The subject invention provides methods which allow the production of soluble *C. botulinum* toxin proteins in economical host cells (*e.g.*, *E. coli*). In addition the subject invention provides methods which allow the production of soluble botulinal toxin proteins in yeast and insect cells. Further, methods for the isolation of purified soluble *C. botulinum*

toxin proteins which are suitable for immunization of humans and other animals are provided. These soluble, purified preparations of *C. botulinum* toxin proteins provide the basis for improved vaccine preparations and facilitate the production of antitoxin.

When recombinant clostridial toxin proteins produced in gram-negative bacteria (*e.g.*, *E. coli*) are used as vaccines, they are purified to remove endotoxin prior to administration to a host animal. In order to vaccinate a host, an immunogenically-effective amount of purified substantially endotoxin-free recombinant clostridial toxin protein is administered in any of a number of physiologically acceptable carriers known to the art. When administered for the purpose of vaccination, the purified substantially endotoxin-free recombinant clostridial toxin protein may be used alone or in conjunction with known adjuvants, including potassium alum, aluminum phosphate, aluminum hydroxide, Gerbu adjuvant (GmDP; C.C. Biotech Corp.), RIBI adjuvant (MPL; RIBI Immunochemical Research, Inc.), QS21 (Cambridge Biotech). The alum and aluminum-based adjuvants are particularly preferred when vaccines are to be administered to humans; however, any adjuvant approved for use in humans may be employed. The route of immunization may be nasal, oral, intramuscular, intraperitoneal or subcutaneous.

The invention contemplates the use of soluble, substantially endotoxin-free preparations of fusion proteins comprising the C fragment of the *C. botulinum* type A, B, C, D, E, F, and G toxin as vaccines. In one embodiment, the vaccine comprises the C fragment of either the *C. botulinum* type A, B, C, D, E, F, or G toxin and a poly-histidine tract (also called a histidine tag). In a particularly preferred embodiment, a fusion protein comprising the histidine tagged C fragment is expressed using the pET series of expression vectors (Novagen). The pET expression system utilizes a vector containing the T7 promoter which encodes the fusion protein and a host cell which can be induced to express the T7 DNA polymerase (*i.e.*, a DE3 host strain). The production of C fragment fusion proteins containing a histidine tract is not limited to the use of a particular expression vector and host strain. Several commercially available expression vectors and host strains can be used to express the C fragment protein sequences as a fusion protein containing a histidine tract (For example, the pQE series (pQE-8, 12, 16, 17, 18, 30, 31, 32, 40, 41, 42, 50, 51, 52, 60 and 70) of expression vectors (Qiagen) which are used with the host strains M15[pREP4] (Qiagen) and SG13009[pREP4] (Qiagen) can be used to express fusion proteins containing six histidine residues at the amino-terminus of the fusion protein). Furthermore a number of commercially available expression vectors which provide a histidine tract also provide a protease cleavage

site between the histidine tract and the protein of interest (*e.g.*, botulinal toxin sequences). Cleavage of the resulting fusion protein with the appropriate protease will remove the histidine tag from the protein of interest (*e.g.*, botulinal toxin sequences) (see Example 28a, *infra*). Removal of the histidine tag may be desirable prior to administration of the recombinant botulinal toxin protein to a subject (*e.g.*, a human).

## VI. Detection Of Toxin

The invention contemplates detecting bacterial toxin in a sample. The term "sample" in the present specification and claims is used in its broadest sense. On the one hand it is meant to include a specimen or culture. On the other hand, it is meant to include both biological and environmental samples.

Biological samples may be animal, including human, fluid, solid (*e.g.*, stool) or tissue; liquid and solid food products and ingredients such as dairy items, vegetables, meat and meat by-products, and waste. Environmental samples include environmental material such as surface matter, soil, water and industrial samples, as well as samples obtained from food and dairy processing instruments, apparatus, equipment, disposable and non-disposable items. These examples are not to be construed as limiting the sample types applicable to the present invention.

The invention contemplates detecting bacterial toxin by a competitive immunoassay method that utilizes recombinant toxin A and toxin B proteins, antibodies raised against recombinant bacterial toxin proteins. A fixed amount of the recombinant toxin proteins are immobilized to a solid support (*e.g.*, a microtiter plate) followed by the addition of a biological sample suspected of containing a bacterial toxin. The biological sample is first mixed with affinity-purified or PEG fractionated antibodies directed against the recombinant toxin protein. A reporter reagent is then added which is capable of detecting the presence of antibody bound to the immobilized toxin protein. The reporter substance may comprise an antibody with binding specificity for the antitoxin attached to a molecule which is used to identify the presence of the reporter substance. If toxin is present in the sample, this toxin will compete with the immobilized recombinant toxin protein for binding to the anti-recombinant antibody thereby reducing the signal obtained following the addition of the reporter reagent. A control is employed where the antibody is not mixed with the sample. This gives the highest (or reference) signal.

The invention also contemplates detecting bacterial toxin by a "sandwich" immunoassay method that utilizes antibodies directed against recombinant bacterial toxin proteins. Affinity-purified antibodies directed against recombinant bacterial toxin proteins are immobilized to a solid support (*e.g.*, microtiter plates). Biological samples suspected of containing bacterial toxins are then added followed by a washing step to remove substantially all unbound antitoxin. The biological sample is next exposed to the reporter substance, which binds to antitoxin and is then washed free of substantially all unbound reporter substance. The reporter substance may comprise an antibody with binding specificity for the antitoxin attached to a molecule which is used to identify the presence of the reporter substance. Identification of the reporter substance in the biological tissue indicates the presence of the bacterial toxin.

It is also contemplated that bacterial toxin be detected by pouring liquids (*e.g.*, soups and other fluid foods and feeds including nutritional supplements for humans and other animals) over immobilized antibody which is directed against the bacterial toxin. It is contemplated that the immobilized antibody will be present in or on such supports as cartridges, columns, beads, or any other solid support medium. In one embodiment, following the exposure of the liquid to the immobilized antibody, unbound toxin is substantially removed by washing. The exposure of the liquid is then exposed to a reporter substance which detects the presence of bound toxin. In a preferred embodiment the reporter substance is an enzyme, fluorescent dye, or radioactive compound attached to an antibody which is directed against the toxin (*i.e.*, in a "sandwich" immunoassay). It is also contemplated that the detection system will be developed as necessary (*e.g.*, the addition of enzyme substrate in enzyme systems; observation using fluorescent light for fluorescent dye systems; and quantitation of radioactivity for radioactive systems).

## EXPERIMENTAL

The following examples serve to illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

In the disclosure which follows, the following abbreviations apply: °C (degrees Centigrade); rpm (revolutions per minute); BBS-Tween (borate buffered saline containing Tween); BSA (bovine serum albumin); ELISA (enzyme-linked immunosorbent assay); CFA (complete Freund's adjuvant); IFA (incomplete Freund's adjuvant); IgG (immunoglobulin G); IgY (immunoglobulin Y); IM (intramuscular); IP (intraperitoneal); IV (intravenous or

intravascular); SC (subcutaneous); H<sub>2</sub>O (water); HCl (hydrochloric acid); LD<sub>100</sub> (lethal dose for 100% of experimental animals); aa (amino acid); HPLC (high performance liquid chromatography); kD (kilodaltons); gm (grams); µg (micrograms); mg (milligrams); ng (nanograms); µl (microliters); ml (milliliters); mm (millimeters); nm (nanometers); µm (micrometer); M (molar); mM (millimolar); MW (molecular weight); sec (seconds); min(s) (minute/minutes); hr(s) (hour/hours); MgCl<sub>2</sub> (magnesium chloride); NaCl (sodium chloride); Na<sub>2</sub>CO<sub>3</sub> (sodium carbonate); OD<sub>280</sub> (optical density at 280 nm); OD<sub>600</sub> (optical density at 600 nm); PAGE (polyacrylamide gel electrophoresis); PBS [phosphate buffered saline (150 mM NaCl, 10 mM sodium phosphate buffer, pH 7.2)]; PEG (polyethylene glycol); PMSF (phenylmethylsulfonyl fluoride); SDS (sodium dodecyl sulfate); Tris (tris(hydroxymethyl)aminomethane); Ensure® (Ensure®, Ross Laboratories, Columbus OH); Enfamil® (Enfamil®, Mead Johnson); w/v (weight to volume); v/v (volume to volume); Amicon (Amicon, Inc., Beverly, MA); Amresco (Amresco, Inc., Solon, OH); ATCC (American Type Culture Collection, Rockville, MD); BBL (Baltimore Biologics Laboratory, a division of Becton Dickinson), Cockeysville, MD); Becton Dickinson (Becton Dickinson Labware, Lincoln Park, NJ); BioRad (BioRad, Richmond, CA); Biotech (C-C Biotech Corp., Poway, CA); Charles River (Charles River Laboratories, Wilmington, MA); Cocalico (Cocalico Biologicals Inc., Reamstown, PA); CytRx (CytRx Corp., Norcross, GA); Falcon (e.g. Baxter Healthcare Corp., McGaw Park, IL and Becton Dickinson); FDA (Federal Food and Drug Administration); Fisher Biotech (Fisher Biotech, Springfield, NJ); GIBCO (Grand Island Biologic Company/BRL, Grand Island, NY); Gibco-BRL (Life Technologies, Inc., Gaithersburg, MD); Harlan Sprague Dawley (Harlan Sprague Dawley, Inc., Madison, WI); Mallinckrodt (a division of Baxter Healthcare Corp., McGaw Park, IL); Millipore (Millipore Corp., Marlborough, MA); New England Biolabs (New England Biolabs, Inc., Beverly, MA); Novagen (Novagen, Inc., Madison, WI); Pharmacia (Pharmacia, Inc., Piscataway, NJ); Qiagen (Qiagen, Chatsworth, CA); Saseo (Saseo, Omaha, NE); Showdex (Showa Denko America, Inc., New York, NY); Sigma (Sigma Chemical Co., St. Louis, MO); Sterogene (Sterogene, Inc., Arcadia, CA); Tech Lab (Tech Lab, Inc., Blacksburg, VA); and Vaxcell (Vaxcell, Inc., a subsidiary of CytRX Corp., Norcross, GA).

When a recombinant protein is described in the specification it is referred to in a short-hand manner by the amino acids in the toxin sequence present in the recombinant protein rounded to the nearest 10. For example, the recombinant protein pMB1850-2360 contains amino acids 1852 through 2362 of the *C. difficile* toxin B protein. The specification

gives detailed construction details for all recombinant proteins such that one skilled in the art will know precisely which amino acids are present in a given recombinant protein.

### EXAMPLE 1

#### Production Of High-Titer Antibodies To *Clostridium difficile* Organisms In A Hen

Antibodies to certain pathogenic organisms have been shown to be effective in treating diseases caused by those organisms. It has not been shown whether antibodies can be raised against *Clostridium difficile*, which would be effective in treating infection by this organism. Accordingly, *C. difficile* was tested as immunogen for production of hen antibodies.

To determine the best course for raising high-titer egg antibodies against whole *C. difficile* organisms, different immunizing strains and different immunizing concentrations were examined. The example involved (a) preparation of the bacterial immunogen, (b) immunization, (c) purification of anti-bacterial chicken antibodies, and (d) detection of anti-bacterial antibodies in the purified IgY preparations.

##### a) Preparation Of Bacterial Immunogen

*C. difficile* strains 43594 (serogroup A) and 43596 (serogroup C) were originally obtained from the ATCC. These two strains were selected because they represent two of the most commonly-occurring serogroups isolated from patients with antibiotic-associated pseudomembranous colitis. [Delmee *et al.*, J. Clin. Microbiol., 28(10):2210 (1990).] Additionally, both of these strains have been previously characterized with respect to their virulence in the Syrian hamster model for *C. difficile* infection. [Delmee *et al.*, J. Med Microbiol., 33:85 (1990).]

The bacterial strains were separately cultured on brain heart infusion agar for 48 hours at 37°C in a Gas Pack 100 Jar (BBL, Cockeysville, MD) equipped with a Gas Pack Plus anaerobic envelope (BBL). Forty-eight hour cultures were used because they produce better growth and the organisms have been found to be more cross-reactive with respect to their surface antigen presentation. The greater the degree of cross-reactivity of our IgY preparations, the better the probability of a broad range of activity against different strains/serogroups. [Toma *et al.*, J. Clin. Microbiol., 26(3):426 (1988).]

The resulting organisms were removed from the agar surface using a sterile dacron-tip swab, and were suspended in a solution containing 0.4% formaldehyde in PBS, pH 7.2. This



concentration of formaldehyde has been reported as producing good results for the purpose of preparing whole-organism immunogen suspensions for the generation of polyclonal anti-*C. difficile* antisera in rabbits. [Delmee *et al.*, J. Clin. Microbiol., 21:323 (1985); Davies *et al.*, Microbial Path., 9:141 (1990).] In this manner, two separate bacterial suspensions were prepared, one for each strain. The two suspensions were then incubated at 4°C for 1 hour. Following this period of formalin-treatment, the suspensions were centrifuged at  $4,200 \times g$  for 20 min., and the resulting pellets were washed twice in normal saline. The washed pellets, which contained formalin-treated whole organisms, were resuspended in fresh normal saline such that the visual turbidity of each suspension corresponded to a #7 McFarland standard. [M.A.C. Edelstein, "Processing Clinical Specimens for Anaerobic Bacteria: Isolation and Identification Procedures," in S.M. Finegold *et al* (eds.), *Bailey and Scott's Diagnostic Microbiology*, pp. 477-507, C.V. Mosby Co., (1990). The preparation of McFarland nephelometer standards and the corresponding approximate number of organisms for each tube are described in detail at pp. 172-173 of this volume.] Each of the two #7 suspensions was then split into two separate volumes. One volume of each suspension was volumetrically adjusted, by the addition of saline, to correspond to the visual turbidity of a #1 McFarland standard. [*Id.*] The #1 suspensions contained approximately  $3 \times 10^8$  organisms/ml, and the #7 suspensions contained approximately  $2 \times 10^9$  organisms/ml. [*Id.*] The four resulting concentration-adjusted suspensions of formalin-treated *C. difficile* organisms were considered to be "bacterial immunogen suspensions." These suspensions were used immediately after preparation for the initial immunization. [See section (b).]

The formalin-treatment procedure did not result in 100% non-viable bacteria in the immunogen suspensions. In order to increase the level of killing, the formalin concentration and length of treatment were both increased for subsequent immunogen preparations, as described below in Table 3. (Although viability was decreased with the stronger formalin treatment, 100% inviability of the bacterial immunogen suspensions was not reached.) Also, in subsequent immunogen preparations, the formalin solutions were prepared in normal saline instead of PBS. At day 49, the day of the fifth immunization, the excess volumes of the four previous bacterial immunogen suspensions were stored frozen at -70°C for use during all subsequent immunizations.

### b) Immunization

For the initial immunization, 1.0 ml volumes of each of the four bacterial immunogen suspensions described above were separately emulsified in 1.2 ml volumes of CFA (GIBCO). For each of the four emulsified immunogen suspensions, two four-month old White Leghorn hens (pre-laying) were immunized. (It is not necessary to use pre-laying hens; actively-laying hens can also be utilized.) Each hen received a total volume of approximately 1.0 ml of a single emulsified immunogen suspension via four injections (two subcutaneous and two intramuscular) of approximately 250  $\mu$ l per site. In this manner, a total of four different immunization combinations, using two hens per combination, were initiated for the purpose of evaluating both the effect of immunizing concentration on egg yolk antibody (IgY) production, and interstrain cross-reactivity of IgY raised against heterologous strains. The four immunization groups are summarized in Table 3.

TABLE 3

Immunization Groups

Group Designation	Immunizing Strain	Approximate Immunizing Dose
CD 43594, #1	<i>C. difficile</i> strain 43594	$1.5 \times 10^8$ organisms/hen
CD 43594, #7	" "	$1.0 \times 10^8$ organisms/hen
CD 43596, #1	<i>C. difficile</i> strain 43596	$1.5 \times 10^8$ organisms/hen
CD 43596, #7	" "	$1.0 \times 10^8$ organisms/hen

The time point for the first series of immunizations was designated as "day zero." All subsequent immunizations were performed as described above except that the bacterial immunogen suspensions were emulsified using IFA (GIBCO) instead of CFA, and for the later time point immunization, the stored frozen suspensions were used instead of freshly-prepared suspensions. The immunization schedule used is listed in Table 4.

**TABLE 4**  
Immunization Schedule

Day Of Immunization	Formalin-Treatment	Immunogen Preparation Used
0	1%, 1 hr.	freshly-prepared
14	1%, overnight	" "
21	1%, overnight	" "
35	1%, 48 hrs.	" "
49	1%, 72 hrs.	" "
70	" "	stored frozen
85	" "	" "
105	" "	" "

**c) Purification Of Anti-Bacterial Chicken Antibodies**

Groups of four eggs were collected per immunization group between days 80 and 84 post-initial immunization, and chicken immunoglobulin (IgY) was extracted according to a modification of the procedure of A. Polson *et al.*, Immunol. Comm., 9:495 (1980). A gentle stream of distilled water from a squirt bottle was used to separate the yolks from the whites, and the yolks were broken by dropping them through a funnel into a graduated cylinder. The four individual yolks were pooled for each group. The pooled, broken yolks were blended with 4 volumes of egg extraction buffer to improve antibody yield (egg extraction buffer is 0.01 M sodium phosphate, 0.1 M NaCl, pH 7.5, containing 0.005% thimerosal), and PEG 8000 (Amresco) was added to a concentration of 3.5%. When all the PEG dissolved, the protein precipitates that formed were pelleted by centrifugation at  $13,000 \times g$  for 10 minutes. The supernatants were decanted and filtered through cheesecloth to remove the lipid layer, and the PEG was added to the supernatants to a final concentration of 12% (the supernatants were assumed to contain 3.5% PEG). After a second centrifugation, the supernatants were discarded and the pellets were centrifuged a final time to extrude the remaining PEG. These crude IgY pellets were then dissolved in the original yolk volume of egg extraction buffer and stored at 4°C. As an additional control, a preimmune IgY solution was prepared as described above, using eggs collected from unimmunized hens.

**d) Detection Of Anti-Bacterial Antibodies In The Purified IgY Preparations**

In order to evaluate the relative levels of specific anti-*C. difficile* activity in the IgY preparations described above, a modified version of the whole-organism ELISA procedure of N.V. Padhye *et al.*, J. Clin. Microbiol. 29:99-103 (1990) was used. Frozen organisms of both *C. difficile* strains described above were thawed and diluted to a concentration of approximately  $1 \times 10^7$  organisms/ml using PBS, pH 7.2. In this way, two separate coating suspensions were prepared, one for each immunizing strain. Into the wells of 96-well microtiter plates (Falcon, Pro-Bind Assay Plates) were placed 100  $\mu$ l volumes of the coating suspensions. In this manner, each plate well received a total of approximately  $1 \times 10^8$  organisms of one strain or the other. The plates were then incubated at 4°C overnight. The next morning, the coating suspensions were decanted, and all wells were washed three times using PBS. In order to block non-specific binding sites, 100  $\mu$ l of 0.5% BSA (Sigma) in PBS was then added to each well, and the plates were incubated for 2 hours at room temperature. The blocking solution was decanted, and 100  $\mu$ l volumes of the IgY preparations described above were initially diluted 1:500 with a solution of 0.1% BSA in PBS, and then serially diluted in 1:5 steps. The following dilutions were placed in the wells: 1:500, 1:2,500, 1:62,500, 1:312,500, and 1:1,562,500. The plates were again incubated for 2 hours at room temperature. Following this incubation, the IgY-containing solutions were decanted, and the wells were washed three times using BBS-Tween (0.1 M boric acid, 0.025 M sodium borate, 1.0 M NaCl, 0.1% Tween-20), followed by two washes using PBS-Tween (0.1% Tween-20), and finally, two washes using PBS only. To each well, 100  $\mu$ l of a 1:750 dilution of rabbit anti-chicken IgG (whole-molecule)-alkaline phosphatase conjugate (Sigma) (diluted in 0.1% BSA in PBS) was added. The plates were again incubated for 2 hours at room temperature. The conjugate solutions were decanted and the plates were washed as described above, substituting 50 mM Na<sub>2</sub>CO<sub>3</sub>, pH 9.5 for the PBS in the final wash. The plates were developed by the addition of 100  $\mu$ l of a solution containing 1 mg/ml para-nitrophenyl phosphate (Sigma) dissolved in 50 mM Na<sub>2</sub>CO<sub>3</sub>, 10 mM MgCl<sub>2</sub>, pH 9.5 to each well, and incubating the plates at room temperature in the dark for 45 minutes. The absorbance of each well was measured at 410 nm using a Dynatech MR 700 plate reader. In this manner, each of the four IgY preparations described above was tested for reactivity against both of the immunizing *C. difficile* strains: strain-specific, as well as cross-reactive activity was determined.

Table 5 shows the results of the whole-organism ELISA. All four IgY preparations demonstrated significant levels of activity, to a dilution of 1:62,500 or greater against both of the immunizing organism strains. Therefore, antibodies raised against one strain were highly cross-reactive with the other strain, and vice versa. The immunizing concentration of organisms did not have a significant effect on organism-specific IgY production, as both concentrations produced approximately equivalent responses. Therefore, the lower immunizing concentration of approximately  $1.5 \times 10^8$  organisms/hen is the preferred immunizing concentration of the two tested. The preimmune IgY preparation appeared to possess relatively low levels of *C. difficile*-reactive activity to a dilution of 1:500, probably due to prior exposure of the animals to environmental clostridia.

An initial whole-organism ELISA was performed using IgY preparations made from single CD 43594, #1 and CD 43596, #1 eggs collected around day 50 (data not shown). Specific titers were found to be 5 to 10-fold lower than those reported in Table 5. These results demonstrate that it is possible to begin immunizing hens prior to the time that they begin to lay eggs, and to obtain high titer specific IgY from the first eggs that are laid. In other words, it is not necessary to wait for the hens to begin laying before the immunization schedule is started.

TABLE 5

Results Of The Anti-*C. difficile* Whole-Organism ELISA

IgY Preparation	Dilution Of IgY Prep	43594-Coated Wells	43596-Coated Wells
CD 43594, #1	1:500	1.746	1.801
	1:2,500	1.092	1.670
	1:12,500	0.202	0.812
	1:62,500	0.136	0.179
	1:312,500	0.012	0.080
	1:1,562,500	0.002	0.020
CD 43594, #7	1:500	1.780	1.771
	1:2,500	1.025	1.078
	1:12,500	0.188	0.382
	1:62,500	0.052	0.132
	1:312,500	0.022	0.043
	1:1,562,500	0.005	0.024
CD 43596, #1	1:500	1.526	1.790
	1:2,500	0.832	1.477
	1:12,500	0.247	0.452
	1:62,500	0.050	0.242
	1:312,500	0.010	0.067
	1:1,562,500	0.000	0.036
CD 43596, #7	1:500	1.702	1.505
	1:2,500	0.706	0.866
	1:12,500	0.250	0.282
	1:62,500	0.039	0.078
	1:312,500	0.002	0.017
	1:1,562,500	0.000	0.010
Preimmune IgY	1:500	0.142	0.309
	1:2,500	0.032	0.077
	1:12,500	0.006	0.024
	1:62,500	0.002	0.012
	1:312,500	0.004	0.010
	1:1,562,500	0.002	0.014

## EXAMPLE 2

Treatment Of *C. difficile* Infection With Anti-*C. difficile* Antibody

In order to determine whether the immune IgY antibodies raised against whole *C. difficile* organisms were capable of inhibiting the infection of hamsters by *C. difficile*, hamsters infected by these bacteria were utilized. [Lyerly *et al.*, Infect. Immun., 59:2215-2218 (1991).] This example involved: (a) determination of the lethal dose of *C. difficile* organisms; and (b) treatment of infected animals with immune antibody or control antibody in nutritional solution.

a) **Determination Of The Lethal Dose Of *C. difficile* Organisms**

Determination of the lethal dose of *C. difficile* organisms was carried out according to the model described by D.M. Lysterly *et al.*, Infect. Immun., 59:2215-2218 (1991). *C. difficile* strain ATCC 43596 (serogroup C, ATCC) was plated on BHI agar and grown anaerobically (BBL Gas Pak 100 system) at 37°C for 42 hours. Organisms were removed from the agar surface using a sterile dacron-tip swab and suspended in sterile 0.9% NaCl solution to a density of  $10^8$  organisms/ml.

In order to determine the lethal dose of *C. difficile* in the presence of control antibody and nutritional formula, non-immune eggs were obtained from unimmunized hens and a 12% PEG preparation made as described in Example 1(c). This preparation was redissolved in one fourth the original yolk volume of vanilla flavor Ensure®.

Starting on day one, groups of female Golden Syrian hamsters (Harlan Sprague Dawley), 8-9 weeks old and weighing approximately 100 gm, were orally administered 1 ml of the preimmune/Ensure® formula at time zero, 2 hours, 6 hours, and 10 hours. At 1 hour, animals were orally administered 3.0 mg clindamycin HCl (Sigma) in 1 ml of water. This drug predisposes hamsters to *C. difficile* infection by altering the normal intestinal flora. On day two, the animals were given 1 ml of the preimmune IgY/Ensure® formula at time zero, 2 hours, 6 hours, and 10 hours. At 1 hour on day two, different groups of animals were inoculated orally with saline (control), or  $10^2$ ,  $10^4$ ,  $10^6$ , or  $10^8$  *C. difficile* organisms in 1 ml of saline. From days 3-12, animals were given 1 ml of the preimmune IgY/Ensure® formula three times daily and observed for the onset of diarrhea and death. Each animal was housed in an individual cage and was offered food and water *ad libitum*.

Administration of  $10^6$  -  $10^8$  organisms resulted in death in 3-4 days while the lower doses of  $10^2$  -  $10^4$  organisms caused death in 5 days. Cecal swabs taken from dead animals indicated the presence of *C. difficile*. Given the effectiveness of the  $10^2$  dose, this number of organisms was chosen for the following experiment to see if hyperimmune anti-*C. difficile* antibody could block infection.

b) **Treatment Of Infected Animals With Immune Antibody Or Control Antibody In Nutritional Formula**

The experiment in (a) was repeated using three groups of seven hamsters each. Group A received no clindamycin or *C. difficile* and was the survival control. Group B received clindamycin,  $10^2$  *C. difficile* organisms and preimmune IgY on the same schedule as the

animals in (a) above. Group C received clindamycin,  $10^2$  *C. difficile* organisms, and hyperimmune anti-*C. difficile* IgY on the same schedule as Group B. The anti-*C. difficile* IgY was prepared as described in Example 1 except that the 12% PEG preparation was dissolved in one fourth the original yolk volume of Ensure®.

All animals were observed for the onset of diarrhea or other disease symptoms and death. Each animal was housed in an individual cage and was offered food and water *ad libitum*. The results are shown in Table 6.

TABLE 6

The Effect Of Oral Feeding Of Hyperimmune IgY Antibody on *C. difficile* Infection

Animal Group	Time To Diarrhea*	Time To Death*
A pre-immune IgY only	no diarrhea	no deaths
B Clindamycin, <i>C. difficile</i> , preimmune IgY	30 hrs.	49 hrs.
C Clindamycin, <i>C. difficile</i> , immune IgY	33 hrs.	56 hrs.

Mean of seven animals.

Hamsters in the control group A did not develop diarrhea and remained healthy during the experimental period. Hamsters in groups B and C developed diarrheal disease. Anti-*C. difficile* IgY did not protect the animals from diarrhea or death, all animals succumbed in the same time interval as the animals treated with preimmune IgY. Thus, while immunization with whole organisms apparently can improve sub-lethal symptoms with particular bacteria (see U.S. Patent No. 5,080,895 to H. Tokoro), such an approach does not prove to be productive to protect against the lethal effects of *C. difficile*.

### EXAMPLE 3

#### Production of *C. botulinum* Type A Antitoxin in Hens

In order to determine whether antibodies could be raised against the toxin produced by clostridial pathogens, which would be effective in treating clostridial diseases, antitoxin to *C. botulinum* type A toxin was produced. This example involves: (a) toxin modification; (b) immunization; (c) antitoxin collection; (d) antigenicity assessment; and (e) assay of antitoxin titer.



a) **Toxin Modification**

*C. botulinum* type A toxoid was obtained from B. R. DasGupta. From this, the active type A neurotoxin (M.W. approximately 150 kD) was purified to greater than 99% purity, according to published methods. [B.R. DasGupta & V. Sathyamoorthy, Toxicon, 22:415 (1984).] The neurotoxin was detoxified with formaldehyde according to published methods. [B.R. Singh & B.R. DasGupta, Toxicon, 27:403 (1989).]

b) **Immunization**

*C. botulinum* toxoid for immunization was dissolved in PBS (1 mg/ml) and was emulsified with an approximately equal volume of CFA (GIBCO) for initial immunization or IFA for booster immunization. On day zero, two white leghorn hens, obtained from local breeders, were each injected at multiple sites (intramuscular and subcutaneous) with 1 ml inactivated toxoid emulsified in 1 ml CFA. Subsequent booster immunizations were made according to the following schedule for day of injection and toxoid amount: days 14 and 21 - 0.5 mg; day 171 - 0.75 mg; days 394, 401, 409 - 0.25 mg. One hen received an additional booster of 0.150 mg on day 544.

c) **Antitoxin Collection**

Total yolk immunoglobulin (IgY) was extracted as described in Example 1(c) and the IgY pellet was dissolved in the original yolk volume of PBS with thimerosal.

d) **Antigenicity Assessment**

Eggs were collected from day 409 through day 423 to assess whether the toxoid was sufficiently immunogenic to raise antibody. Eggs from the two hens were pooled and antibody was collected as described in the standard PEG protocol. [Example 1(c).] Antigenicity of the botulinal toxin was assessed on Western blots. The 150 kD detoxified type A neurotoxin and unmodified, toxic, 300 kD botulinal type A complex (toxin used for intragastric route administration for animal gut neutralization experiments; see Example 6) were separated on a SDS-polyacrylamide reducing gel. The Western blot technique was performed according to the method of Towbin. [H. Towbin *et al.*, Proc. Natl. Acad. Sci. USA, 76:4350 (1979).] Ten µg samples of *C. botulinum* complex and toxoid were dissolved in SDS reducing sample buffer (1% SDS, 0.5% 2-mercaptoethanol, 50 mM Tris, pH 6.8, 10% glycerol, 0.025% w/v bromphenol blue, 10% β-mercaptoethanol), heated at 95°C for 10 min

and separated on a 1 mm thick 5% SDS-polyacrylamide gel. [K. Weber and M. Osborn, "Proteins and Sodium Dodecyl Sulfate: Molecular Weight Determination on Polyacrylamide Gels and Related Procedures," in *The Proteins*, 3d Edition (H. Neurath & R.L. Hill, eds), pp. 179-223, (Academic Press, NY, 1975).] Part of the gel was cut off and the proteins were stained with Coomassie Blue. The proteins in the remainder of the gel were transferred to nitrocellulose using the Milliblot-SDE electro-blotting system (Millipore) according to manufacturer's directions. The nitrocellulose was temporarily stained with 10% Ponceau S [S.B. Carroll and A. Laughon, "Production and Purification of Polyclonal Antibodies to the Foreign Segment of  $\beta$ -galactosidase Fusion Proteins," in *DNA Cloning: A Practical Approach*, Vol.III, (D. Glover, ed.), pp. 89-111, IRL Press, Oxford, (1987)] to visualize the lanes, then destained by running a gentle stream of distilled water over the blot for several minutes. The nitrocellulose was immersed in PBS containing 3% BSA overnight at 4°C to block any remaining protein binding sites.

The blot was cut into strips and each strip was incubated with the appropriate primary antibody. The avian anti-*C. botulinum* antibodies [described in (c)] and pre-immune chicken antibody (as control) were diluted 1:125 in PBS containing 1 mg/ml BSA for 2 hours at room temperature. The blots were washed with two changes each of large volumes of PBS, BBS-Tween and PBS, successively (10 min/wash). Goat anti-chicken IgG alkaline phosphatase conjugated secondary antibody (Fisher Biotech) was diluted 1:500 in PBS containing 1 mg/ml BSA and incubated with the blot for 2 hours at room temperature. The blots were washed with two changes each of large volumes of PBS and BBS-Tween, followed by one change of PBS and 0.1 M Tris-HCl, pH 9.5. Blots were developed in freshly prepared alkaline phosphatase substrate buffer (100  $\mu$ g/ml nitroblue tetrazolium (Sigma), 50  $\mu$ g/ml 5-bromo-4-chloro-3-indolyl phosphate (Sigma), 5 mM  $MgCl_2$  in 50 mM  $Na_2CO_3$ , pH 9.5).

The Western blots are shown in Figure 1. The anti-*C. botulinum* IgY reacted to the toxoid to give a broad immunoreactive band at about 145-150 kD on the reducing gel. This toxoid is refractive to disulfide cleavage by reducing agents due to formalin crosslinking. The immune IgY reacted with the active toxin complex, a 97 kD *C. botulinum* type A heavy chain and a 53 kD light chain. The preimmune IgY was unreactive to the *C. botulinum* complex or toxoid in the Western blot.

**c) Antitoxin Antibody Titer**

The IgY antibody titer to *C. botulinum* type A toxoid of eggs harvested between day 409 and 423 was evaluated by ELISA, prepared as follows. Ninety-six-well Falcon Pro-bind plates were coated overnight at 4°C with 100 µl/well toxoid [B.R. Singh & B.R. Das Gupta, Toxicon 27:403 (1989)] at 2.5 µg/ml in PBS, pH 7.5 containing 0.005% thimerosal. The following day the wells were blocked with PBS containing 1% BSA for 1 hour at 37°C. The IgY from immune or preimmune eggs was diluted in PBS containing 1% BSA and 0.05% Tween 20 and the plates were incubated for 1 hour at 37°C. The plates were washed three times with PBS containing 0.05% Tween 20 and three times with PBS alone. Alkaline phosphatase-conjugated goat-anti-chicken IgG (Fisher Biotech) was diluted 1:750 in PBS containing 1% BSA and 0.05% Tween 20, added to the plates, and incubated 1 hour at 37°C. The plates were washed as before, and p-nitrophenyl phosphate (Sigma) at 1 mg/ml in 0.05 M Na<sub>2</sub>CO<sub>3</sub>, pH 9.5, 10 mM MgCl<sub>2</sub> was added.

The results are shown in Figure 2. Chickens immunized with the toxoid generated high titers of antibody to the immunogen. Importantly, eggs from both immunized hens had significant anti-immunogen antibody titers as compared to preimmune control eggs. The anti-*C. botulinum* IgY possessed significant activity, to a dilution of 1:93,750 or greater.

**EXAMPLE 4**

**Preparation Of Avian Egg Yolk Immunoglobulin In An Orally Administrable Form**

In order to administer avian IgY antibodies orally to experimental mice, an effective delivery formula for the IgY had to be determined. The concern was that if the crude IgY was dissolved in PBS, the saline in PBS would dehydrate the mice, which might prove harmful over the duration of the study. Therefore, alternative methods of oral administration of IgY were tested. The example involved: (a) isolation of immune IgY; (b) solubilization of IgY in water or PBS, including subsequent dialysis of the IgY-PBS solution with water to eliminate or reduce the salts (salt and phosphate) in the buffer; and (c) comparison of the quantity and activity of recovered IgY by absorbance at 280 nm and PAGE, and enzyme-linked immunoassay (ELISA).

a) **Isolation Of Immune IgY**

In order to investigate the most effective delivery formula for IgY, we used IgY which was raised against *Crotalus durissus terrificus* venom. Three eggs were collected from hens immunized with the *C. durissus terrificus* venom and IgY was extracted from the yolks using the modified Polson procedure described by Thalley and Carroll [Bio/Technology, 8:934-938 (1990)] as described in Example 1(c).

The egg yolks were separated from the whites, pooled, and blended with four volumes of PBS. Powdered PEG 8000 was added to a concentration of 3.5%. The mixture was centrifuged at 10,000 rpm for 10 minutes to pellet the precipitated protein, and the supernatant was filtered through cheesecloth to remove the lipid layer. Powdered PEG 8000 was added to the supernatant to bring the final PEG concentration to 12% (assuming a PEG concentration of 3.5% in the supernatant). The 12% PEG/IgY mixture was divided into two equal volumes and centrifuged to pellet the IgY.

b) **Solubilization Of The IgY In Water Or PBS**

One pellet was resuspended in 1/2 the original yolk volume of PBS, and the other pellet was resuspended in 1/2 the original yolk volume of water. The pellets were then centrifuged to remove any particles or insoluble material. The IgY in PBS solution dissolved readily but the fraction resuspended in water remained cloudy.

In order to satisfy anticipated sterility requirements for orally administered antibodies, the antibody solution needs to be filter-sterilized (as an alternative to heat sterilization which would destroy the antibodies). The preparation of IgY resuspended in water was too cloudy to pass through either a 0.2 or 0.45  $\mu\text{m}$  membrane filter, so 10 ml of the PBS resuspended fraction was dialyzed overnight at room temperature against 250 ml of water. The following morning the dialysis chamber was emptied and refilled with 250 ml of fresh  $\text{H}_2\text{O}$  for a second dialysis. Thereafter, the yields of soluble antibody were determined at  $\text{OD}_{280}$  and are compared in Table 7.

TABLE 7

Dependence Of IgY Yield On Solvents

Fraction	Absorbance Of 1:10 Dilution At 280 nm	Percent Recovery
PBS dissolved	1.149	100%
H <sub>2</sub> O dissolved	0.706	61%
PBS dissolved/H <sub>2</sub> O dialyzed	0.885	77%

Resuspending the pellets in PBS followed by dialysis against water recovered more antibody than directly resuspending the pellets in water (77% versus 61%). Equivalent volumes of the IgY preparation in PBS or water were compared by PAGE, and these results were in accordance with the absorbance values (data not shown).

#### c) Activity Of IgY Prepared With Different Solvents

An ELISA was performed to compare the binding activity of the IgY extracted by each procedure described above. *C. durissus terrificus* (C.d.t.) venom at 2.5 µg/ml in PBS was used to coat each well of a 96-well microtiter plate. The remaining protein binding sites were blocked with PBS containing 5 mg/ml BSA. Primary antibody dilutions (in PBS containing 1 mg/ml BSA) were added in duplicate. After 2 hours of incubation at room temperature, the unbound primary antibodies were removed by washing the wells with PBS, BBS-Tween, and PBS. The species specific secondary antibody (goat anti-chicken immunoglobulin alkaline-phosphatase conjugate (Sigma) was diluted 1:750 in PBS containing 1 mg/ml BSA and added to each well of the microtiter plate. After 2 hours of incubation at room temperature, the unbound secondary antibody was removed by washing the plate as before, and freshly prepared alkaline phosphatase substrate (Sigma) at 1 mg/ml in 50 mM Na<sub>2</sub>CO<sub>3</sub>, 10 mM MgCl<sub>2</sub>, pH 9.5 was added to each well. The color development was measured on a Dynatech MR 700 microplate reader using a 412 nm filter. The results are shown in Table 8.

The binding assay results parallel the recovery values in Table 7, with PBS-dissolved IgY showing slightly more activity than the PBS-dissolved/H<sub>2</sub>O dialyzed antibody. The water-dissolved antibody had considerably less binding activity than the other preparations.

**EXAMPLE 5****Survival Of Antibody Activity After Passage Through The Gastrointestinal Tract**

In order to determine the feasibility of oral administration of antibody, it was of interest to determine whether orally administered IgY survived passage through the gastrointestinal tract. The example involved: (a) oral administration of specific immune antibody mixed with a nutritional formula; and (b) assay of antibody activity extracted from feces.

**TABLE 8**

Antigen-Binding Activity Of IgY Prepared With Different Solvents

Dilution	Preimmune	PBS Dissolved	H <sub>2</sub> O Dissolved	PBS/H <sub>2</sub> O
1:500	0.005	1.748	1.577	1.742
1:2,500	0.004	0.644	0.349	0.606
1:12,500	0.001	0.144	0.054	0.090
1:62,500	0.001	0.025	0.007	0.016
1:312,500	0.010	0.000	0.000	0.002

**a) Oral Administration Of Antibody**

The IgY preparations used in this example are the same PBS-dissolved/H<sub>2</sub>O dialyzed antivenom materials obtained in Example 4 above, mixed with an equal volume of Enfamil®. Two mice were used in this experiment, each receiving a different diet as follows:

- 1) water and food as usual;
  - 2) immune IgY preparation dialyzed against water and mixed 1:1 with Enfamil®.
- (The mice were given the corresponding mixture as their only source of food and water).

**b) Antibody Activity After Ingestion**

After both mice had ingested their respective fluids, each tube was refilled with approximately 10 ml of the appropriate fluid first thing in the morning. By mid-morning there was about 4 to 5 ml of liquid left in each tube. At this point stool samples were collected from each mouse, weighed, and dissolved in approximately 500  $\mu$ l PBS per 100 mg stool sample. One hundred and sixty mg of control stools (no antibody) and 99 mg of experimental stools (specific antibody) in 1.5 ml microfuge tubes were dissolved in 800 and 500  $\mu$ l PBS, respectively. The samples were heated at 37°C for 10 minutes and vortexed vigorously. The experimental stools were also broken up with a narrow spatula. Each sample

was centrifuged for 5 minutes in a microfuge and the supernatants, presumably containing the antibody extracts, were collected. The pellets were saved at 2-8°C in case future extracts were needed. Because the supernatants were tinted, they were diluted five-fold in PBS containing 1 mg/ml BSA for the initial dilution in the enzyme immunoassay (ELISA). The primary extracts were then diluted five-fold serially from this initial dilution. The volume of primary extract added to each well was 190 µl. The ELISA was performed exactly as described in Example 4.

TABLE 9

Specific Antibody Activity After Passage Through The Gastrointestinal Tract

Dilution	Preimmune IgY	Control Fecal Extract	EXP. Fecal Extract
1:5	0	0.000	0.032
1:25	0.016	0	0.016
1:125	0	0	0.009
1:625	0	0.003	0.001
1:3125	0	0	0.000

There was some active antibody in the fecal extract from the mouse given the specific antibody in Enfamil® formula, but it was present at a very low level. Since the samples were assayed at an initial 1:5 dilution, the binding observed could have been higher with less dilute samples. Consequently, the mice were allowed to continue ingesting either regular food and water or the specific IgY in Enfamil® formula, as appropriate, so the assay could be repeated. Another ELISA plate was coated overnight with 5 µg/ml of *C.d.t.* venom in PBS.

The following morning the ELISA plate was blocked with 5 mg/ml BSA, and the fecal samples were extracted as before, except that instead of heating the extracts at 37°C, the samples were kept on ice to limit proteolysis. The samples were assayed undiluted initially, and in 5X serial dilutions thereafter. Otherwise the assay was carried out as before.

TABLE 10

Specific Antibody Survives Passage Through The Gastrointestinal Tract

Dilution	Preimmune IgY	Control Extract	Exp. Extract
undiluted	0.003	· 0	0.379
1:5	· 0	· 0	0.071
1:25	0.000	· 0	0.027
1:125	0.003	· 0	0.017
1:625	0.000	· 0	0.008
1:3125	0.002	· 0	0.002

The experiment confirmed the previous results, with the antibody activity markedly higher. The control fecal extract showed no anti-*C. d.t.* activity, even undiluted, while the fecal extract from the anti-*C. d.t.* IgY/Enfamil®-fed mouse showed considerable anti-*C. d.t.* activity. This experiment (and the previous experiment) clearly demonstrate that active IgY antibody survives passage through the mouse digestive tract, a finding with favorable implications for the success of IgY antibodies administered orally as a therapeutic or prophylactic.

### EXAMPLE 6

#### *In Vivo* Neutralization Of Type C. botulinum Type A Neurotoxin By Avian Antitoxin Antibody

This example demonstrated the ability of PEG-purified antitoxin, collected as described in Example 3, to neutralize the lethal effect of *C. botulinum* neurotoxin type A in mice. To determine the oral lethal dose (LD<sub>100</sub>) of toxin A, groups of BALB/c mice were given different doses of toxin per unit body weight (average body weight of 24 grams). For oral administration, toxin A complex, which contains the neurotoxin associated with other non-toxin proteins was used. This complex is markedly more toxic than purified neurotoxin when given by the oral route. [I. Ohishi *et al.*, Infect. Immun., 16:106 (1977).] *C. botulinum* toxin type A complex, obtained from Eric Johnson (University Of Wisconsin, Madison) was 250 µg/ml in 50 mM sodium citrate, pH 5.5, specific toxicity  $3 \times 10^7$  mouse LD<sub>50</sub>/mg with parenteral administration. Approximately 40-50 ng/gm body weight was usually fatal within 48 hours in mice maintained on conventional food and water. When mice were given a diet *ad libitum* of only Enfamil® the concentration needed to produce lethality was approximately



2.5 times higher (125 ng/gm body weight). Botulinal toxin concentrations of approximately 200 ng/gm body weight were fatal in mice fed Enfamil® containing preimmune IgY (resuspended in Enfamil® at the original yolk volume).

The oral LD<sub>100</sub> of *C. botulinum* toxin was also determined in mice that received known amounts of a mixture of preimmune IgY-Ensure® delivered orally through feeding needles. Using a 22 gauge feeding needle, mice were given 250 µl each of a preimmune IgY-Ensure® mixture (preimmune IgY dissolved in 1/4 original yolk volume) 1 hour before and 1/2 hour and 5 hours after administering botulinal toxin. Toxin concentrations given orally ranged from approximately 12 to 312 ng/gm body weight (0.3 to 7.5 µg per mouse). Botulinal toxin complex concentration of approximately 40 ng/gm body weight (1 µg per mouse) was lethal in all mice in less than 36 hours.

Two groups of BALB/c mice, 10 per group, were each given orally a single dose of 1 µg each of botulinal toxin complex in 100 µl of 50 mM sodium citrate pH 5.5. The mice received 250 µl treatments of a mixture of either preimmune or immune IgY in Ensure® (1/4 original yolk volume) 1 hour before and 1/2 hour, 4 hours, and 8 hours after botulinal toxin administration. The mice received three treatments per day for two more days. The mice were observed for 96 hours. The survival and mortality are shown in Table 11.

TABLE 11

Neutralization Of Botulinal Toxin A *In Vivo*

Toxin Dose ng/gm	Antibody Type	Number Of Mice Alive	Number Of Mice Dead
41.6	non-immune	0	10
41.6	anti-botulinal toxin	10	0

All mice treated with the preimmune IgY-Ensure® mixture died within 46 hours post-toxin administration. The average time of death in the mice was 32 hours post toxin administration. Treatments of preimmune IgY-Ensure® mixture did not continue beyond 24 hours due to extensive paralysis of the mouth in mice of this group. In contrast, all ten mice treated with the immune anti-botulinal toxin IgY-Ensure® mixture survived past 96 hours. Only 4 mice in this group exhibited symptoms of botulism toxicity (two mice about 2 days after and two mice 4 days after toxin administration). These mice eventually died 5 and 6 days later. Six of the mice in this immune group displayed no adverse effects to the toxin and remained alive and healthy long term. Thus, the avian anti-botulinal toxin antibody demonstrated very good protection from the lethal effects of the toxin in the experimental mice.

**EXAMPLE 7****Production Of An Avian Antitoxin Against *Clostridium difficile* Toxin A**

Toxin A is a potent cytotoxin secreted by pathogenic strains of *C. difficile*, that plays a direct role in damaging gastrointestinal tissues. In more severe cases of *C. difficile* intoxication, pseudomembranous colitis can develop which may be fatal. This would be prevented by neutralizing the effects of this toxin in the gastrointestinal tract. As a first step, antibodies were produced against a portion of the toxin. The example involved: (a) conjugation of a synthetic peptide of toxin A to bovine serum albumin; (b) immunization of hens with the peptide-BSA conjugate; and (c) detection of antitoxin peptide antibodies by ELISA.

**a) Conjugation Of A Synthetic Peptide Of Toxin A To Bovine Serum Albumin**

The synthetic peptide CQTIDGKKYYFN-NH<sub>2</sub> (SEQ ID NO:82) was prepared commercially (Multiple Peptide Systems, San Diego, CA) and validated to be >80% pure by high-pressure liquid chromatography. The eleven amino acids following the cysteine residue represent a consensus sequence of a repeated amino acid sequence found in Toxin A. [Wren *et al.*, Infect. Immun., 59:3151-3155 (1991).] The cysteine was added to facilitate conjugation to carrier protein.

In order to prepare the carrier for conjugation, BSA (Sigma) was dissolved in 0.01 M NaPO<sub>4</sub>, pH 7.0 to a final concentration of 20 mg/ml and n-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS; Pierce) was dissolved in N,N-dimethyl formamide to a concentration of 5 mg/ml. MBS solution, 0.51 ml, was added to 3.25 ml of the BSA solution and incubated for 30 minutes at room temperature with stirring every 5 minutes. The MBS-activated BSA was then purified by chromatography on a Bio-Gel P-10 column (Bio-Rad; 40 ml bed volume) equilibrated with 50 mM NaPO<sub>4</sub>, pH 7.0 buffer. Peak fractions were pooled (6.0 ml).

Lyophilized toxin A peptide (20 mg) was added to the activated BSA mixture, stirred until the peptide dissolved and incubated 3 hours at room temperature. Within 20 minutes, the reaction mixture became cloudy and precipitates formed. After 3 hours, the reaction mixture was centrifuged at 10,000 × g for 10 min and the supernatant analyzed for protein content. No significant protein could be detected at 280 nm. The conjugate precipitate was

washed three times with PBS and stored at 4°C. A second conjugation was performed with 15 mg of activated BSA and 5 mg of peptide and the conjugates pooled and suspended at a peptide concentration of 10 mg/ml in 10 mM NaPO<sub>4</sub>, pH 7.2.

#### b) Immunization Of Hens With Peptide Conjugate

Two hens were each initially immunized on day zero by injection into two subcutaneous and two intramuscular sites with 1 mg of peptide conjugate that was emulsified in CFA (GIBCO). The hens were boosted on day 14 and day 21 with 1 mg of peptide conjugate emulsified in IFA (GIBCO).

#### c) Detection Of Antitoxin Peptide Antibodies By ELISA

IgY was purified from two eggs obtained before immunization (pre-immune) and two eggs obtained 31 and 32 days after the initial immunization using PEG fractionation as described in Example 1.

Wells of a 96-well microtiter plate (Falcon Pro-Bind Assay Plate) were coated overnight at 4°C with 100 µg/ml solution of the toxin A synthetic peptide in PBS, pH 7.2 prepared by dissolving 1 mg of the peptide in 1.0 ml of H<sub>2</sub>O and dilution of PBS. The pre-immune and immune IgY preparations were diluted in a five-fold series in a buffer containing 1% PEG 8000 and 0.1% Tween-20 (v/v) in PBS, pH 7.2. The wells were blocked for 2 hours at room temperature with 150 µl of a solution containing 5% (v/v) Carnation® nonfat dry milk and 1% PEG 8000 in PBS, pH 7.2. After incubation for 2 hours at room temperature, the wells were washed, secondary rabbit anti-chicken IgG-alkaline phosphatase (1:750) added, the wells washed again and the color development obtained as described in Example 1. The results are shown in Table 12.

TABLE 12

Reactivity Of IgY With Toxin Peptide

Dilution Of PEG Prep	Absorbance At 410 nm	
	Preimmune	Immune Anti-Peptide
1:100	0.013	0.253
1:500	0.004	0.039
1:2500	0.004	0.005

Clearly, the immune antibodies contain titers against this repeated epitope of toxin A.

**EXAMPLE 8****Production Of Avian Antitoxins Against  
*Clostridium difficile* Native Toxins A And B**

To determine whether avian antibodies are effective for the neutralization of *C. difficile* toxins, hens were immunized using native *C. difficile* toxins A and B. The resulting egg yolk antibodies were then extracted and assessed for their ability to neutralize toxins A and B *in vitro*. The Example involved (a) preparation of the toxin immunogens, (b) immunization, (c) purification of the antitoxins, and (d) assay of toxin neutralization activity.

**a) Preparation Of The Toxin Immunogens**

Both *C. difficile* native toxins A and B, and *C. difficile* toxoids, prepared by the treatment of the native toxins with formaldehyde, were employed as immunogens. *C. difficile* toxoids A and B were prepared by a procedure which was modified from published methods (Ehrich *et al.*, Infect. Immun. 28:1041 (1980). Separate solutions (in PBS) of native *C. difficile* toxin A and toxin B (Tech Lab) were each adjusted to a concentration of 0.20 mg/ml, and formaldehyde was added to a final concentration of 0.4%. The toxin/formaldehyde solutions were then incubated at 37°C for 40 hrs. Free formaldehyde was then removed from the resulting toxoid solutions by dialysis against PBS at 4°C. In previously published reports, this dialysis step was not performed. Therefore, free formaldehyde must have been present in their toxoid preparations. The toxoid solutions were concentrated, using a Centriprep concentrator unit (Amicon), to a final toxoid concentration of 4.0 mg/ml. The two resulting preparations were designated as toxoid A and toxoid B.

*C. difficile* native toxins were prepared by concentrating stock solutions of toxin A and toxin B (Tech Lab, Inc), using Centriprep concentrator units (Amicon), to a final concentration of 4.0 mg/ml.

**b) Immunization**

The first two immunizations were performed using the toxoid A and toxoid B immunogens described above. A total of 3 different immunization combinations were employed. For the first immunization group, 0.2 ml of toxoid A was emulsified in an equal volume of Titer Max adjuvant (CytRx). Titer Max was used in order to conserve the amount of immunogen used, and to simplify the immunization procedure. This immunization group

was designated "CTA." For the second immunization group, 0.1 ml of toxoid B was emulsified in an equal volume of Titer Max adjuvant. This group was designated "CTB." For the third immunization group, 0.2 ml of toxoid A was first mixed with 0.2 ml of toxoid B, and the resulting mixture was emulsified in 0.4 ml of Titer Max adjuvant. This group was designated "CTAB." In this way, three separate immunogen emulsions were prepared, with each emulsion containing a final concentration of 2.0 mg/ml of toxoid A (CTA) or toxoid B (CTB) or a mixture of 2.0 mg/ml toxoid A and 2.0 mg/ml toxoid B (CTAB).

On day 0, White Leghorn hens, obtained from a local breeder, were immunized as follows: **Group CTA.** Four hens were immunized, with each hen receiving 200µg of toxoid A, via two intramuscular (I.M.) injections of 50µl of CTA emulsion in the breast area. **Group CTB.** One hen was immunized with 200µg of toxoid B, via two I.M. injections of 50µl of CTB emulsion in the breast area. **Group CTAB.** Four hens were immunized, with each hen receiving a mixture containing 200µg of toxoid A and 200µg of toxoid B, via two I.M. injections of 100µl of CTAB emulsion in the breast area. The second immunization was performed 5 weeks later, on day 35, exactly as described for the first immunization above.

In order to determine whether hens previously immunized with *C. difficile* toxoids could tolerate subsequent booster immunizations using native toxins, a single hen from group CTAB was immunized for a third time, this time using a mixture of the native toxin A and native toxin B described in section (a) above (these toxins were not formaldehyde-treated, and were used in their active form). This was done in order to increase the amount (titer) and affinity of specific antitoxin antibody produced by the hen over that achieved by immunizing with toxoids only. On day 62, 0.1 ml of a toxin mixture was prepared which contained 200µg of native toxin A and 200µg of native toxin B. This toxin mixture was then emulsified in 0.1 ml of Titer Max adjuvant. A single CTAB hen was then immunized with the resulting immunogen emulsion, via two I.M. injections of 100µl each, into the breast area. This hen was marked with a wing band, and observed for adverse effects for a period of approximately 1 week, after which time the hen appeared to be in good health.

Because the CTAB hen described above tolerated the booster immunization with native toxins A and B with no adverse effects, it was decided to boost the remaining hens with native toxin as well. On day 70, booster immunizations were performed as follows: **Group CTA.** A 0.2 ml volume of the 4 mg/ml native toxin A solution was emulsified in an equal volume of Titer Max adjuvant. Each of the 4 hens was then immunized with 200µg of native toxin A, as described for the toxoid A immunizations above. **Group CTB.** A 50µl volume

of the 4 mg/ml native toxin B solution was emulsified in an equal volume of Titer Max adjuvant. The hen was then immunized with 200µg of native toxin B, as described for the toxoid B immunizations above. **Group CTAB.** A 0.15 ml volume of the 4 mg/ml native toxin A solution was first mixed with a 0.15 ml volume the 4 mg/ml native toxin B solution. The resulting toxin mixture was then emulsified in 0.3 ml of Titer Max adjuvant. The 3 remaining hens (the hen with the wing band was not immunized this time) were then immunized with 200µg of native toxin A and 200µg of native toxin B as described for the toxoid A+ toxoid B immunizations (CTAB) above. On day 85, all hens received a second booster immunization using native toxins, done exactly as described for the first boost with native toxins above.

All hens tolerated both booster immunizations with native toxins with no adverse effects. As previous literature references describe the use of formaldehyde-treated toxoids, this is apparently the first time that any immunizations have been performed using native *C. difficile* toxins.

#### c) Purification Of Antitoxins

Eggs were collected from the hen in group CTB 10-12 days following the second immunization with toxoid (day 35 immunization described in section (b) above), and from the hens in groups CTA and CTAB 20-21 days following the second immunization with toxoid. To be used as a pre-immune (negative) control, eggs were also collected from unimmunized hens from the same flock. Egg yolk immunoglobulin (IgY) was extracted from the 4 groups of eggs as described in Example 1 (c), and the final IgY pellets were solubilized in the original yolk volume of PBS without thimerosal. Importantly, thimerosal was excluded because it would have been toxic to the CHO cells used in the toxin neutralization assays described in section (d) below.

#### d) Assay Of Toxin Neutralization Activity

The toxin neutralization activity of the IgY solutions prepared in section (c) above was determined using an assay system that was modified from published methods. [Ehrich *et al.*, Infect. Immun. 28:1041-1043 (1992); and McGee *et al.* Microb. Path. 12:333-341 (1992).] As additional controls, affinity-purified goat anti-*C. difficile* toxin A (Tech Lab) and affinity-purified goat anti-*C. difficile* toxin B (Tech Lab) were also assayed for toxin neutralization activity.

The IgY solutions and goat antibodies were serially diluted using F 12 medium (GIBCO) which was supplemented with 2% FCS (GIBCO)(this solution will be referred to as "medium" for the remainder of this Example). The resulting antibody solutions were then mixed with a standardized concentration of either native *C. difficile* toxin A (Tech Lab), or native *C. difficile* toxin B (Tech Lab), at the concentrations indicated below. Following incubation at 37°C for 60 min., 100µl volumes of the toxin + antibody mixtures were added to the wells of 96-well microtiter plates (Falcon Microtest III) which contained  $2.5 \times 10^4$  Chinese Hamster Ovary (CHO) cells per well (the CHO cells were plated on the previous day to allow them to adhere to the plate wells). The final concentration of toxin, or dilution of antibody indicated below refers to the final test concentration of each reagent present in the respective microtiter plate wells. Toxin reference wells were prepared which contained CHO cells and toxin A or toxin B at the same concentration used for the toxin plus antibody mixtures (these wells contained no antibody). Separate control wells were also prepared which contained CHO cells and medium only. The assay plates were then incubated for 18-24 hrs. in a 37°C, humidified, 5% CO<sub>2</sub> incubator. On the following day, the remaining adherent (viable) cells in the plate wells were stained using 0.2% crystal violet (Mallinckrodt) dissolved in 2% ethanol, for 10 min. Excess stain was then removed by rinsing with water, and the stained cells were solubilized by adding 100µl of 1% SDS (dissolved in water) to each well. The absorbance of each well was then measured at 570 nm, and the percent cytotoxicity of each test sample or mixture was calculated using the following formula:

$$\% \text{ CHO Cell Cytotoxicity} = \left[ 1 - \left( \frac{\text{Abs. Sample}}{\text{Abs. Control}} \right) \right] \times 100$$

Unlike previous reports which quantitate results visually by counting cell rounding by microscopy, this Example utilized spectrophotometric methods to quantitate the *C. difficile* toxin bioassay. In order to determine the toxin A neutralizing activity of the CTA, CTAB, and pre-immune IgY preparations, as well as the affinity-purified goat antitoxin A control, dilutions of these antibodies were reacted against a 0.1µg/ml concentration of native toxin A (this is the approx. 50% cytotoxic dose of toxin A in this assay system). The results are shown in Figure 3.

Complete neutralization of toxin A occurred with the CTA IgY (antitoxin A, above) at dilutions of 1:80 and lower, while significant neutralization occurred out to the 1:320 dilution.

The CTAB IgY (antitoxin A + toxin B, above) demonstrated complete neutralization at the 1:320-1:160 and lower dilutions, and significant neutralization occurred out to the 1:1280 dilution. The commercially available affinity-purified goat antitoxin A did not completely neutralize toxin A at any of the dilutions tested, but demonstrated significant neutralization out to a dilution of 1:1,280. The preimmune IgY did not show any toxin A neutralizing activity at any of the concentrations tested. These results demonstrate that IgY purified from eggs laid by hens immunized with toxin A alone, or simultaneously with toxin A and toxin B, is an effective toxin A antitoxin.

The toxin B neutralizing activity of the CTAB and pre-immune IgY preparations, and also the affinity-purified goat antitoxin B control was determined by reacting dilutions of these antibodies against a concentration of native toxin B of 0.1 ng/ml (approximately the 50% cytotoxic dose of toxin B in the assay system). The results are shown in Figure 4.

Complete neutralization of toxin B occurred with the CTAB IgY (antitoxin A + toxin B, above) at the 1:40 and lower dilutions, and significant neutralization occurred out to the 1:320 dilution. The affinity-purified goat antitoxin B demonstrated complete neutralization at dilutions of 1:640 and lower, and significant neutralization occurred out to a dilution of 1:2,560. The preimmune IgY did not show any toxin B neutralizing activity at any of the concentrations tested. These results demonstrate that IgY purified from eggs laid by hens immunized simultaneously with toxin A and toxin B is an effective toxin B antitoxin.

In a separate study, the toxin B neutralizing activity of CTB, CTAB, and pre-immune IgY preparations was determined by reacting dilutions of these antibodies against a native toxin B concentration of 0.1 µg/ml (approximately 100% cytotoxic dose of toxin B in this assay system). The results are shown in Figure 5.

Significant neutralization of toxin B occurred with the CTB IgY (antitoxin B, above) at dilutions of 1:80 and lower, while the CTAB IgY (antitoxin A + toxin B, above) was found to have significant neutralizing activity at dilutions of 1:40 and lower. The preimmune IgY did not show any toxin B neutralizing activity at any of the concentrations tested. These results demonstrate that IgY purified from eggs laid by hens immunized with toxin B alone, or simultaneously with toxin A and toxin B, is an effective toxin B antitoxin.



**EXAMPLE 9**

*In vivo* Protection Of Golden Syrian Hamsters From  
*C. difficile* Disease By Avian Antitoxins Against *C. difficile* Toxins A And B

5       The most extensively used animal model to study *C. difficile* disease is the hamster. [Lyerly *et al.*, Infect. Immun. 47:349-352 (1992).] Several other animal models for antibiotic-induced diarrhea exist, but none mimic the human form of the disease as closely as the hamster model. [R. Fekety, "Animal Models of Antibiotic-Induced Colitis," in O. Zak and M. Sande (eds.), *Experimental Models in Antimicrobial Chemotherapy*, Vol. 2, pp.61-72, 10       (1986).] In this model, the animals are first predisposed to the disease by the oral administration of an antibiotic, such as clindamycin, which alters the population of normally-occurring gastrointestinal flora (Fekety, at 61-72). Following the oral challenge of these animals with viable *C. difficile* organisms, the hamsters develop cecitis, and hemorrhage, ulceration, and inflammation are evident in the intestinal mucosa. [Lyerly *et al.*, Infect. 15       Immun. 47:349-352 (1985).] The animals become lethargic, develop severe diarrhea, and a high percentage of them die from the disease. [Lyerly *et al.*, Infect. Immun. 47:349-352 (1985).] This model is therefore ideally suited for the evaluation of therapeutic agents designed for the treatment or prophylaxis of *C. difficile* disease.

20       The ability of the avian *C. difficile* antitoxins, described in Example 1 above, to protect hamsters from *C. difficile* disease was evaluated using the Golden Syrian hamster model of *C. difficile* infection. The Example involved (a) preparation of the avian *C. difficile* antitoxins, (b) *in vivo* protection of hamsters from *C. difficile* disease by treatment with avian antitoxins, and (c) long-term survival of treated hamsters.

25       **a)       Preparation Of The Avian *C. difficile* Antitoxins**

30       Eggs were collected from hens in groups CTA and CTAB described in Example 1 (b) above. To be used as a pre-immune (negative) control, eggs were also purchased from a local supermarket. Egg yolk immunoglobulin (IgY) was extracted from the 3 groups of eggs as described in Example 1 (c), and the final IgY pellets were solubilized in one fourth the original yolk volume of Ensure-8 nutritional formula.

b) ***In vivo* Protection Of Hamsters Against *C. difficile* Disease By  
Treatment With Avian Antitoxins**

The avian *C. difficile* antitoxins prepared in section (a) above were evaluated for their ability to protect hamsters from *C. difficile* disease using an animal model system which was modified from published procedures. [Fekety, at 61-72; Borriello *et al.*, J. Med. Microbiol., 24:53-64 (1987); Kim *et al.*, Infect. Immun., 55:2984-2992 (1987); Borriello *et al.*, J. Med. Microbiol., 25:191-196 (1988); Delmee and Avesani, J. Med. Microbiol., 33:85-90 (1990); and Lysterly *et al.*, Infect. Immun., 59:2215-2218 (1991).] For the study, three separate experimental groups were used, with each group consisting of 7 female Golden Syrian hamsters (Charles River), approximately 10 weeks old and weighing approximately 100 gms. each. The three groups were designated "CTA," "CTAB" and "Pre-immune." These designations corresponded to the antitoxin preparations with which the animals in each group were treated. Each animal was housed in an individual cage, and was offered food and water *ad libitum* through the entire length of the study. On day 1, each animal was orally administered 1.0 ml of one of the three antitoxin preparations (prepared in section (a) above) at the following timepoints: 0 hrs., 4 hrs., and 8 hrs. On day 2, the day 1 treatment was repeated. On day 3, at the 0 hr. timepoint, each animal was again administered antitoxin, as described above. At 1 hr., each animal was orally administered 3.0 mg of clindamycin-HCl (Sigma) in 1 ml of water. This treatment predisposed the animals to infection with *C. difficile*. As a control for possible endogenous *C. difficile* colonization, an additional animal from the same shipment (untreated) was also administered 3.0 mg of clindamycin-HCl in the same manner. This clindamycin control animal was left untreated (and uninfected) for the remainder of the study. At the 4 hr. and 8 hr. timepoints, the animals were administered antitoxin as described above. On day 4, at the 0 hr. timepoint, each animal was again administered antitoxin as described above. At 1 hr., each animal was orally challenged with 1 ml of *C. difficile* inoculum, which contained approx. 100 *C. difficile* strain 43596 organisms in sterile saline. *C. difficile* strain 43596, which is a serogroup C strain, was chosen because it is representative of one of the most frequently-occurring serogroups isolated from patients with antibiotic-associated pseudomembranous colitis. [Delmee *et al.*, J. Clin. Microbiol., 28:2210-2214 (1985).] In addition, this strain has been previously demonstrated to be virulent in the hamster model of infection. [Delmee and Avesani, J. Med. Microbiol., 33:85-90 (1990).] At the 4 hr. and 8 hr. timepoints, the animals were administered antitoxin as described above. On days 5 through 13, the animals were administered antitoxin 3x per day

as described for day 1 above, and observed for the onset of diarrhea and death. On the morning of day 14, the final results of the study were tabulated. These results are shown in Table 13.

Representative animals from those that died in the Pre-Immune and CTA groups were necropsied. Viable *C. difficile* organisms were cultured from the ceca of these animals, and the gross pathology of the gastrointestinal tracts of these animals was consistent with that expected for *C. difficile* disease (inflamed, distended, hemorrhagic cecum, filled with watery diarrhea-like material). In addition, the clindamycin control animal remained healthy throughout the entire study period, therefore indicating that the hamsters used in the study had not previously been colonized with endogenous *C. difficile* organisms prior to the start of the study. Following the final antitoxin treatment on day 13, a single surviving animal from the CTA group, and also from the CTAB group, was sacrificed and necropsied. No pathology was noted in either animal.

TABLE 13

Treatment Results

Treatment Group	No. Animals Surviving	No. Animals Dead
Pre-Immune	1	6
CTA (Antitoxin A only)	5	2
CTAB (Antitoxin A + Antitoxin B)	7	0

Treatment of hamsters with orally-administered toxin A and toxin B antitoxin (group CTAB) successfully protected 7 out of 7 (100%) of the animals from *C. difficile* disease. Treatment of hamsters with orally-administered toxin A antitoxin (group CTA) protected 5 out of 7 (71%) of these animals from *C. difficile* disease. Treatment using pre-immune IgY was not protective against *C. difficile* disease, as only 1 out of 7 (14%) of these animals survived. These results demonstrate that the avian toxin A antitoxin and the avian toxin A + toxin B antitoxin effectively protected the hamsters from *C. difficile* disease. These results also suggest that although the neutralization of toxin A alone confers some degree of protection against *C. difficile* disease, in order to achieve maximal protection, simultaneous antitoxin A and antitoxin B activity is necessary.

### c) Long-Term Survival Of Treated Hamsters

It has been previously reported in the literature that hamsters treated with orally-administered bovine antitoxin IgG concentrate are protected from *C. difficile* disease as long

as the treatment is continued, but when the treatment is stopped, the animals develop diarrhea and subsequently die within 72 hrs. [Lyerly *et al.*, Infect. Immun., 59(6):2215-2218 (1991).]

In order to determine whether treatment of *C. difficile* disease using avian antitoxins promotes long-term survival following the discontinuation of treatment, the 4 surviving  
5 animals in group CTA, and the 6 surviving animals in group CTAB were observed for a period of 11 days (264 hrs.) following the discontinuation of antitoxin treatment described in section (b) above. All hamsters remained healthy through the entire post-treatment period. This result demonstrates that not only does treatment with avian antitoxin protect against the onset of *C. difficile* disease (*i.e.*, it is effective as a prophylactic), it also promotes long-term  
10 survival beyond the treatment period, and thus provides a lasting cure.

#### EXAMPLE 10

##### *In vivo* Treatment Of Established *C. difficile* Infection In Golden Syrian Hamsters With Avian Antitoxins Against *C. difficile* Toxins A And B

The ability of the avian *C. difficile* antitoxins, described in Example 8 above, to treat an established *C. difficile* infection was evaluated using the Golden Syrian hamster model. The Example involved (a) preparation of the avian *C. difficile* antitoxins, (b) *in vivo* treatment of hamsters with established *C. difficile* infection, and (c) histologic evaluation of cecal tissue.  
20

##### a) Preparation Of The Avian *C. difficile* Antitoxins

Eggs were collected from hens in group CTAB described in Example 8 (b) above, which were immunized with *C. difficile* toxoids and native toxins A and B. Eggs purchased from a local supermarket were used as a pre-immune (negative) control. Egg yolk  
25 immunoglobulin (IgY) was extracted from the 2 groups of eggs as described in Example 1 (c), and the final IgY pellets were solubilized in one-fourth the original yolk volume of Ensure® nutritional formula.

##### b) *In vivo* Treatment Of Hamsters With Established *C. difficile* Infection

The avian *C. difficile* antitoxins prepared in section (a) above were evaluated for the ability to treat established *C. difficile* infection in hamsters using an animal model system  
30

which was modified from the procedure which was described for the hamster protection study in Example 8(b) above.

For the study, four separate experimental groups were used, with each group consisting of 7 female Golden Syrian hamsters (Charles River), approx. 10 weeks old, weighing approximately 100 gms. each. Each animal was housed separately, and was offered food and water *ad libitum* through the entire length of the study.

On day 1 of the study, the animals in all four groups were each predisposed to *C. difficile* infection by the oral administration of 3.0 mg of clindamycin-HCl (Sigma) in 1 ml of water.

On day 2, each animal in all four groups was orally challenged with 1 ml of *C. difficile* inoculum, which contained approximately 100 *C. difficile* strain 43596 organisms in sterile saline. *C. difficile* strain 43596 was chosen because it is representative of one of the most frequently-occurring serogroups isolated from patients with antibiotic-associated pseudomembranous colitis. [Delmee *et al.*, J. Clin. Microbiol., 28:2210-2214 (1990).] In addition, as this was the same *C. difficile* strain used in all of the previous Examples above, it was again used in order to provide experimental continuity.

On day 3 of the study (24 hrs. post-infection), treatment was started for two of the four groups of animals. Each animal of one group was orally administered 1.0 ml of the CTAB IgY preparation (prepared in section (a) above) at the following timepoints: 0 hrs., 4 hrs., and 8 hrs. The animals in this group were designated "CTAB-24." The animals in the second group were each orally administered 1.0 ml of the pre-immune IgY preparation (also prepared in section (a) above) at the same timepoints as for the CTAB group. These animals were designated "Pre-24." Nothing was done to the remaining two groups of animals on day 3.

On day 4, 48 hrs. post-infection, the treatment described for day 3 above was repeated for the CTAB-24 and Pre-24 groups, and was initiated for the remaining two groups at the same timepoints. The final two groups of animals were designated "CTAB-48" and "Pre-48" respectively.

On days 5 through 9, the animals in all four groups were administered antitoxin or pre-immune IgY, 3x per day, as described for day 4 above. The four experimental groups are summarized in Table 14.

TABLE 14

## Experimental Treatment Groups

Group Designation	Experimental Treatment
CTAB-24	Infected, treatment w/antitoxin IgY started @ 24 hrs. post-infection.
Pre-24	Infected, treatment w/pre-immune IgY started @ 24 hrs. post-infection.
CTAB-48	Infected, treatment w/antitoxin IgY started @ 48 hrs. post-infection.
Pre-48	Infected, treatment w/pre-immune IgY started @ 48 hrs. post-infection.

All animals were observed for the onset of diarrhea and death through the conclusion of the study on the morning of day 10. The results of this study are displayed in Table 15.

TABLE 15

## Experimental Outcome--Day 10

Treatment Group	No. Animals Surviving	No. Animals Dead
CTAB-24	6	1
Pre-24	0	7
CTAB-48	4	3
Pre-48	2	5

Eighty-six percent of the animals which began receiving treatment with antitoxin IgY at 24 hrs. post-infection (CTAB-24 above) survived, while 57% of the animals treated with antitoxin IgY starting 48 hrs. post-infection (CTAB-48 above) survived. In contrast, none of the animals receiving pre-immune IgY starting 24 hrs. post-infection (Pre-24 above) survived, and only 29% of the animals which began receiving treatment with pre-immune IgY at 48 hrs. post-infection (Pre-48 above) survived through the conclusion of the study. These results demonstrate that avian antitoxins raised against *C. difficile* toxins A and B are capable of successfully treating established *C. difficile* infections *in vivo*.

### c) Histologic Evaluation Of Cecal Tissue

In order to further evaluate the ability of the IgY preparations tested in this study to treat established *C. difficile* infection, histologic evaluations were performed on cecal tissue specimens obtained from representative animals from the study described in section (b) above.

Immediately following death, cecal tissue specimens were removed from animals which died in the Pre-24 and Pre-48 groups. Following the completion of the study, a representative surviving animal was sacrificed and cecal tissue specimens were removed from

the CTAB-24 and CTAB-48 groups. A single untreated animal from the same shipment as those used in the study was also sacrificed and a cecal tissue specimen was removed as a normal control. All tissue specimens were fixed overnight at 4°C in 10% buffered formalin. The fixed tissues were paraffin-embedded, sectioned, and mounted on glass microscope slides. The tissue sections were then stained using hematoxylin and eosin (H and E stain), and were examined by light microscopy.

Upon examination, the tissues obtained from the CTAB-24 and CTAB-48 animals showed no pathology, and were indistinguishable from the normal control. This observation provides further evidence for the ability of avian antitoxins raised against *C. difficile* toxins A and B to effectively treat established *C. difficile* infection, and to prevent the pathologic consequences which normally occur as a result of *C. difficile* disease.

In contrast, characteristic substantial mucosal damage and destruction was observed in the tissues of the animals from the Pre-24 and Pre-48 groups which died from *C. difficile* disease. Normal tissue architecture was obliterated in these two preparations, as most of the mucosal layer was observed to have sloughed away, and there were numerous large hemorrhagic areas containing massive numbers of erythrocytes.

## EXAMPLE 11

### Cloning And Expression Of *C. difficile* Toxin A Fragments

The toxin A gene has been cloned and sequenced, and shown to encode a protein of predicted MW of 308 kd. [Dove *et al.*, Infect. Immun., 58:480-488 (1990).] Given the expense and difficulty of isolating native toxin A protein, it would be advantageous to use simple and inexpensive procaryotic expression systems to produce and purify high levels of recombinant toxin A protein for immunization purposes. Ideally, the isolated recombinant protein would be soluble in order to preserve native antigenicity, since solubilized inclusion body proteins often do not fold into native conformations. To allow ease of purification, the recombinant protein should be expressed to levels greater than 1 mg/liter of *E. coli* culture.

To determine whether high levels of recombinant toxin A protein can be produced in *E. coli*, fragments of the toxin A gene were cloned into various prokaryotic expression vectors, and assessed for the ability to express recombinant toxin A protein in *E. coli*. Three prokaryotic expression systems were utilized. These systems were chosen because they drive expression of either fusion (pMALc and pGEX2T) or native (pET23a-c) protein to high levels

in *E. coli*, and allow affinity purification of the expressed protein on a ligand containing column. Fusion proteins expressed from pGEX vectors bind glutathione agarose beads, and are eluted with reduced glutathione. pMAL fusion proteins bind amylose resin, and are eluted with maltose. A poly-histidine tag is present at either the N-terminal (pET16b) or C-terminal (pET23a-c) end of pET fusion proteins. This sequence specifically binds  $\text{Ni}_2^+$  chelate columns, and is eluted with imidazole salts. Extensive descriptions of these vectors are available [Williams *et al.* (1995) *DNA Cloning 2: Expression Systems*, Glover and Hames, eds. IRL Press, Oxford, pp. 15-58], and will not be discussed in detail here. The Example involved (a) cloning of the toxin A gene, (b) expression of large fragments of toxin A in various prokaryotic expression systems, (c) identification of smaller toxin A gene fragments that express efficiently in *E. coli*, (d) purification of recombinant toxin A protein by affinity chromatography, and (e) demonstration of functional activity of a recombinant fragment of the toxin A gene.

#### a) Cloning Of The Toxin A Gene

A restriction map of the toxin A gene is shown in Figure 6. The encoded protein contains a carboxy terminal ligand binding region, containing multiple repeats of a carbohydrate binding domain. [von Eichel-Streiber and Sauerborn, *Gene* 96:107-113 (1990).] The toxin A gene was cloned in three pieces, by using either the polymerase chain reaction (PCR) to amplify specific regions, (regions 1 and 2, Figure 6) or by screening a constructed genomic library for a specific toxin A gene fragment (region 3, Figure 6). The sequences of the utilized PCR primers are P1: 5' GGAAATT TAGCTGCAGCATCTGAC 3' (SEQ ID NO.:1); P2: 5' TCTAGCAAATTCGCTTGT GTTGAA 3' (SEQ ID NO.:2); P3: 5' CTCGCATATAGCATTAGACC 3' (SEQ ID NO.:3); and P4: 5' CTATCTAGGCCTAAAGTAT 3' (SEQ ID NO.:4). These regions were cloned into prokaryotic expression vectors that express either fusion (pMAL.c and pGEX2T) or native (pET23a-c) protein to high levels in *E. coli*, and allow affinity purification of the expressed protein on a ligand containing column.

*Clostridium difficile* VPI strain 10463 was obtained from the ATCC (ATCC #43255) and grown under anaerobic conditions in brain-heart infusion medium (BBL). High molecular-weight *C. difficile* DNA was isolated essentially as described by Wren and Tabaqchali (1987) *J. Clin. Microbiol.*, 25:2402, except proteinase K and sodium dodecyl sulfate (SDS) was used to disrupt the bacteria, and cetyltrimethylammonium bromide



precipitation [as described in Ausubel *et al.*, *Current Protocols in Molecular Biology* (1989)] was used to remove carbohydrates from the cleared lysate. The integrity and yield of genomic DNA was assessed by comparison with a serial dilution of uncut lambda DNA after electrophoresis on an agarose gel.

5        Fragments 1 and 2 were cloned by PCR, utilizing a proofreading thermostable DNA polymerase (native *pfi* polymerase; Stratagene). The high fidelity of this polymerase reduces the mutation problems associated with amplification by error prone polymerases (*e.g.*, *Taq* polymerase). PCR amplification was performed using the indicated PCR primers (Figure 6) in 50 µl reactions containing 10 mM Tris-HCl(8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 200 µM each dNTP, 0.2 µM each primer, and 50 ng *C. difficile* genomic DNA. Reactions were  
10 overlaid with 100 µl mineral oil, heated to 94°C for 4 min, 0.5 µl native *pfi* polymerase (Stratagene) added, and the reaction cycled 30x at 94°C for 1 min, 50°C for 1 min, 72°C for 4 min, followed by 10 min at 72°C. Duplicate reactions were pooled, chloroform extracted, and ethanol precipitated. After washing in 70% ethanol, the pellets were resuspended in 50 µl  
15 TE buffer [10 mM Tris-HCl, 1 mM EDTA pH 8.0]. Aliquots of 10µl each were restriction digested with either *EcoRI/HincII* (fragment 1) or *EcoRI/PstI* (fragment 2), and the appropriate restriction fragments were gel purified using the Prep-A-Gene kit (BioRad), and ligated to either *EcoRI/SmaI*-restricted pGEX2T (Pharmacia) vector (fragment 1), or the *EcoRI/PstI* pMALc (New England Biolabs) vector (fragment 2). Both clones are predicted to  
20 produce in-frame fusions with either the glutathione-S-transferase protein (pGEX vector) or the maltose binding protein (pMAL vector). Recombinant clones were isolated, and confirmed by restriction digestion, using standard recombinant molecular biology techniques. [Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual* (1989), and designated pGA30-660 and pMA660-1100, respectively (*see* Figure 6 for description of the clone designations).]

25        Fragment 3 was cloned from a genomic library of size selected *PstI* digested *C. difficile* genomic DNA, using standard molecular biology techniques (Sambrook *et al.*). Given that the fragment 3 internal *PstI* site is protected from cleavage in *C. difficile* genomic DNA [Price *et al.*, *Curr. Microbiol.*, 16:55-60 (1987)], a 4.7 kb fragment from *PstI* restricted *C. difficile* genomic DNA was gel purified, and ligated to *PstI* restricted, phosphatase treated  
30 pUC9 DNA. The resulting genomic library was screened with a oligonucleotide primer specific to fragment 3, and multiple independent clones were isolated. The presence of fragment 3 in several of these clones was confirmed by restriction digestion, and a clone of the indicated orientation (Figure 6) was restricted with *BamHI/HindIII*, the released fragment

purified by gel electrophoresis, and ligated into similarly restricted pET23c expression vector DNA (Novagen). Recombinant clones were isolated, and confirmed by restriction digestion. This construct is predicted to create both a predicted in frame fusion with the pET protein leader sequence, as well as a predicted C-terminal poly-histidine affinity tag, and is designated pPA1100-2680 (see Figure 6 for the clone designation).

#### b) Expression Of Large Fragments Of Toxin A In *E. coli*

Protein expression from the three expression constructs made in (a) was induced, and analyzed by Western blot analysis with an affinity purified, goat polyclonal antiserum directed against the toxin A toxoid (Tech Lab). The procedures utilized for protein induction, SDS-PAGE, and Western blot analysis are described in detail in Williams *et al* (1995), *supra*. In brief, 5 ml 2X YT (16 g tryptone, 10 g yeast extract, 5 g NaCl per liter, pH 7.5 + 100 µg/ml ampicillin were added to cultures of bacteria (BL21 for pMA1 and pGEX plasmids, and BL21(DE3)lysS for pET plasmids) containing the appropriate recombinant clone which were induced to express recombinant protein by addition of IPTG to 1 mM. Cultures were grown at 37°C, and induced when the cell density reached 0.5 OD<sub>600</sub>. Induced protein was allowed to accumulate for two hrs after induction. Protein samples were prepared by pelleting 1 ml aliquots of bacteria by centrifugation (1 min in a microfuge), and resuspension of the pelleted bacteria in 150 µl of 2x SDS-PAGE sample buffer [Williams *et al.* (1995), *supra*]. The samples were heated to 95°C for 5 min, the cooled and 5 or 10 µl aliquots loaded on 7.5% SDS-PAGE gels. BioRad high molecular weight protein markers were also loaded, to allow estimation of the MW of identified fusion proteins. After electrophoresis, protein was detected either generally by staining gels with Coomassie blue, or specifically, by blotting to nitrocellulose for Western blot detection of specific immunoreactive protein. Western blots, (performed as described in Example 3) which detect toxin A reactive protein in cell lysates of induced protein from the three expression constructs are shown in Figure 7. In this figure, lanes 1-3 contain cell lysates prepared from *E. coli* strains containing pPA1100-2860 in B121(DE3)lysE cells; lanes 4-6 contain cell lysates prepared from *E. coli* strains containing pPA1100-2860 in B121(DE3)lysS cells; lanes 7-9 contain cell lysates prepared from *E. coli* strains containing pMA30-660; lanes 10-12 contain cell lysates prepared from *E. coli* strains containing pMA660-1100. The lanes were probed with an affinity purified goat antitoxin A polyclonal antibody (Tech Lab). Control lysates from uninduced cells (lanes 1, 7, and 10) contain very little immunoreactive material compared to the induced samples in the remaining

lanes. The highest molecular weight band observed for each clone is consistent with the predicted size of the full length fusion protein.

Each construct directs expression of high molecular weight (HMW) protein that is reactive with the toxin A antibody. The size of the largest immunoreactive bands from each sample is consistent with predictions of the estimated MW of the intact fusion proteins. This demonstrates that the three fusions are in-frame, and that none of the clones contain cloning artifacts that disrupt the integrity of the encoded fusion protein. However, the Western blot demonstrates that fusion protein from the two larger constructs (pGA30-660 and pPA1100-2680) are highly degraded. Also, expression levels of toxin A proteins from these two constructs are low, since induced protein bands are not visible by Coomassie staining (not shown). Several other expression constructs that fuse large sub-regions of the toxin A gene to either pMALc or pET23a-c expression vectors, were constructed and tested for protein induction. These constructs were made by mixing gel purified restriction fragments, derived from the expression constructs shown in Figure 6, with appropriately cleaved expression vectors, ligating, and selecting recombinant clones in which the toxin A restriction fragments had ligated together and into the expression vector as predicted for in-frame fusions. The expressed toxin A interval within these constructs are shown in Figure 8, as well as the internal restriction sites utilized to make these constructs.

As used herein, the term "interval" refers to any portion (*i.e.*, any segment of the toxin which is less than the whole toxin molecule) of a clostridial toxin. In a preferred embodiment, "interval" refers to portions of *C. difficile* toxins such as toxin A or toxin B. It is also contemplated that these intervals will correspond to epitopes of immunologic importance, such as antigens or immunogens against which a neutralizing antibody response is effected. It is not intended that the present invention be limited to the particular intervals or sequences described in these Examples. It is also contemplated that sub-portions of intervals (*e.g.*, an epitope contained within one interval or which bridges multiple intervals) be used as compositions and in the methods of the present invention.

In all cases, Western blot analysis of each of these constructs with goat antitoxin A antibody (Tech Lab) detected HMW fusion protein of the predicted size (not shown). This confirms that the reading frame of each of these clones is not prematurely terminated, and is fused in the correct frame with the fusion partner. However, the Western blot analysis revealed that in all cases, the induced protein is highly degraded, and, as assessed by the absence of identifiable induced protein bands by Coomassie Blue staining, are expressed only

at low levels. These results suggest that expression of high levels of intact toxin A recombinant protein is not possible when large regions of the toxin A gene are expressed in *E. coli* using these expression vectors.

5           **c)     High Level Expression Of Small Toxin A Protein Fusions In**  
              ***E. coli***

Experience indicates that expression difficulties are often encountered when large (greater than 100 kd) fragments are expressed in *E. coli*. A number of expression constructs containing smaller fragments of the toxin A gene were constructed, to determine if small  
10 regions of the gene can be expressed to high levels without extensive protein degradation. A summary of these expression constructs are shown in Figure 9. All were constructed by in-frame fusions of convenient toxin A restriction fragments to either the pMAL.c or pET23a-c vectors. Protein preparations from induced cultures of each of these constructs were analyzed by both Coomassie Blue staining and Western analysis as in (b) above. In all cases, higher  
15 levels of intact, full length fusion proteins were observed than with the larger recombinants from section (b).

**d)     Purification Of Recombinant Toxin A Protein**

Large scale (500 ml) cultures of each recombinant from (c) were grown, induced, and  
20 soluble and insoluble protein fractions were isolated. The soluble protein extracts were affinity chromatographed to isolate recombinant fusion protein, as described [Williams *et al.* (1994), *supra*]. In brief, extracts containing tagged pET fusions were chromatographed on a nickel chelate column, and eluted using imidazole salts as described by the distributor (Novagen). Extracts containing soluble pMAL fusion protein were prepared and  
25 chromatographed in column buffer (10 mM NaPO<sub>4</sub>, 0.5M NaCl, 10 mM β-mercaptoethanol, pH 7.2) over an amylose resin column (New England Biolabs), and eluted with column buffer containing 10 mM maltose as described [Williams *et al.* (1995), *supra*]. When the expressed protein was found to be predominantly insoluble, insoluble protein extracts were prepared by the method described in Example 17, *infra*. The results are summarized in Table  
30 16. Figure 10 shows the sample purifications of recombinant toxin A protein. In this figure, lanes 1 and 2 contain MBP fusion protein purified by affinity purification of soluble protein.

TABLE 16

## Purification Of Recombinant Toxin A Protein

Clone <sup>(a)</sup>	Protein Solubility	Yield Affinity Purified Soluble Protein <sup>(b)</sup>	% Intact Soluble Fusion Protein <sup>(c)</sup>	Yield Intact Insoluble Fusion Protein
pMA30-270	Soluble	4 mg/500 mls	10%	NA
pMA30-300	Soluble	4 mg/500 mls	5-10%	NA
pMA300-660	Insoluble	—	NA	10 mg/500 ml
pMA660-1100	Soluble	4.5 mg/500 mls	50%	NA
pMA1100-1610	Soluble	18 mg/500 mls	10%	NA
pMA1610-1870	Both	22 mg/500 mls	90%	20 mg/500 ml
pMA1450-1870	Insoluble	-----	NA	0.2 mg/500 ml
pPA1100-1450	Soluble	0.1 mg/500 mls	90%	NA
pPA1100-1870	Soluble	0.02 mg/500 mls	90%	NA
pMA1870-2680	Both	12 mg/500 mls	80%	NA
pPA1870-2680	Insoluble	-----	NA	10 mg/500 ml

<sup>(a)</sup> pP = pET23 vector, pM=pMAL.c vector, A=toxin A.

<sup>(b)</sup> Based on 1.5 OD<sub>250</sub> = 1 mg/ml (extinction coefficient of MBP).

<sup>(c)</sup> Estimated by Coomassie staining of SDS-PAGE gels.

Lanes 3 and 4 contain MBP fusion protein purified by solubilization of insoluble inclusion bodies. The purified fusion protein samples are pMA1870-2680 (lane 1), pMA660-1100 (lane 2), pMA300-600 (lane 3) and pMA1450-1870 (lane 4).

Poor yields of affinity purified protein were obtained when poly-histidine tagged pET vectors were used to drive expression (pPA1100-1450, pP1100-1870). However, significant protein yields were obtained from pMAL expression constructs spanning the entire toxin A gene, and yields of full-length soluble fusion protein ranged from an estimated 200-400 µg/500 ml culture (pMA30-300) to greater than 20 mg/500 ml culture (pMA1610-1870). Only one interval was expressed to high levels as strictly insoluble protein (pMA300-660). Thus, although high level expression was not observed when using large expression constructs from the toxin A gene, usable levels of recombinant protein spanning the entire toxin A gene were obtainable by isolating induced protein from a series of smaller pMAL expression constructs that span the entire toxin A gene. This is the first demonstration of the feasibility of expressing recombinant toxin A protein to high levels in *E. coli*.

c) **Hemagglutination Assay Using The Toxin A Recombinant Proteins**

The carboxy terminal end consisting of the repeating units contains the hemagglutination activity or binding domain of *C. difficile* toxin A. To determine whether the expressed toxin A recombinants retain functional activity, hemagglutination assays were performed. Two toxin A recombinant proteins, one containing the binding domain as either soluble affinity purified protein (pMA1870-2680) or SDS solubilized inclusion body protein (pPA1870-2680) and soluble protein from one region outside that domain (pMA1100-1610) were tested using a described procedure. [H.C. Krivan *et. al.*, Infect. Immun., 53:573 (1986).] Citrated rabbit red blood cells (RRBC)(Cocalico) were washed several times with Tris-buffer ( 0.1M Tris and 50 mM NaCl ) by centrifugation at 450 x g for 10 minutes at 4° C. A 1% RRBC suspension was made from the packed cells and resuspended in Tris-buffer. Dilutions of the recombinant proteins and native toxin A (Tech Labs) were made in the Tris-buffer and added in duplicate to a round-bottomed 96-well microtiter plate in a final volume of 100 µl. To each well, 50 µl of the 1% RRBC suspension was added, mixed by gentle tapping, and incubated at 4°C for 3-4 hours. Significant hemagglutination occurred only in the recombinant proteins containing the binding domain (pMA 1870-2680) and native toxin A. The recombinant protein outside the binding domain (pMA 1100-1610) displayed no hemagglutination activity. Using equivalent protein concentrations, the hemagglutination titer for toxin A was 1:256, while titers for the soluble and insoluble recombinant proteins of the binding domain were 1:256 and about 1:5000. Clearly, the recombinant proteins tested retained functional activity and were able to bind RRBC's.

**EXAMPLE 12**

**Functional Activity Of IgY Reactive Against Toxin A Recombinants**

The expression of recombinant toxin A protein as multiple fragments in *E.coli* has demonstrated the feasibility of generating toxin A antigen through use of recombinant methodologies (Example 11). The isolation of these recombinant proteins allows the immunoreactivity of each individual subregion of the toxin A protein to be determined (*i.e.*, in a antibody pool directed against the native toxin A protein). This identifies the regions (if any) for which little or no antibody response is elicited when the whole protein is used as a immunogen. Antibodies directed against specific fragments of the toxin A protein can be

purified by affinity chromatography against recombinant toxin A protein, and tested for neutralization ability. This identifies any toxin A subregions that are essential for producing neutralizing antibodies. Comparison with the levels of immune response directed against these intervals when native toxin is used as an immunogen predicts whether potentially higher  
5 titers of neutralizing antibodies can be produced by using recombinant protein directed against a individual region, rather than the entire protein. Finally, since it is unknown whether antibodies reactive to the recombinant toxin A proteins produced in Example 11 neutralize toxin A as effectively as antibodies raised against native toxin A (Examples 9 and 10), the protective ability of a pool of antibodies affinity purified against recombinant toxin A  
10 fragments was assessed for its ability to neutralize toxin A.

This Example involved (a) epitope mapping of the toxin A protein to determine the titre of specific antibodies directed against individual subregions of the toxin A protein when native toxin A protein is used as an immunogen, (b) affinity purification of IgY reactive against recombinant proteins spanning the toxin A gene, (c) toxin A neutralization assays with  
15 affinity purified IgY reactive to recombinant toxin A protein to identify subregions of the toxin A protein that induce the production of neutralizing antibodies, and determination of whether complete neutralization of toxin A can be elicited with a mixture of antibodies reactive to recombinant toxin A protein.

#### 20 a) Epitope Mapping Of The Toxin A Gene

The affinity purification of recombinant toxin A protein specific to defined intervals of the toxin A protein allows epitope mapping of antibody pools directed against native toxin A. This has not previously been possible, since previous expression of toxin A recombinants has been assessed only by Western blot analysis, without knowledge of the expression levels of  
25 the protein [e.g., von Eichel-Streiber *et al.* J. Gen. Microbiol., 135:55-64 (1989)]. Thus, high or low reactivity of recombinant toxin A protein on Western blots may reflect protein expression level differences, not immunoreactivity differences. Given that the purified recombinant protein generated in Example 11 have been quantitated, the issue of relative immunoreactivity of individual regions of the toxin A protein was precisely addressed.

30 For the purposes of this Example, the toxin A protein was subdivided into 6 intervals (1-6), numbered from the amino (interval 1) to the carboxyl (interval 6) termini.

The recombinant proteins corresponding to these intervals were from expression clones (see Example 11(d) for clone designations) pMA30-300 (interval 1), pMA300-660 (interval

2). pMA660-1100 (interval 3), pPA1100-1450 (interval 4), pMA1450-1870 (interval 5) and pMA1870-2680 (interval 6). These 6 clones were selected because they span the entire protein from amino acids numbered 30 through 2680, and subdivide the protein into 6 small intervals. Also, the carbohydrate binding repeat interval is contained specifically in one interval (interval 6), allowing evaluation of the immune response specifically directed against this region. Western blots of 7.5% SDS-PAGE gels, loaded and electrophoresed with defined quantities of each recombinant protein, were probed with either goat antitoxin A polyclonal antibody (Tech Lab) or chicken antitoxin A polyclonal antibody [pCTA IgY, Example 8(c)]. The blots were prepared and developed with alkaline phosphatase as previously described [Williams *et al.* (1995), *supra*]. At least 90% of all reactivity, in either goat or chicken antibody pools, was found to be directed against the ligand binding domain (interval 6). The remaining immunoreactivity was directed against all five remaining intervals, and was similar in both antibody pools, except that the chicken antibody showed a much lower reactivity against interval 2 than the goat antibody.

This clearly demonstrates that when native toxin A is used as an immunogen in goats or chickens, the bulk of the immune response is directed against the ligand binding domain of the protein, with the remaining response distributed throughout the remaining 2/3 of the protein.

#### **b) Affinity Purification Of IgY Reactive Against Recombinant Toxin A Protein**

Affinity columns, containing recombinant toxin A protein from the 6 defined intervals in (a) above, were made and used to (i) affinity purify antibodies reactive to each individual interval from the CTA IgY preparation [Example 8(c)], and (ii) deplete interval specific antibodies from the CTA IgY preparation. Affinity columns were made by coupling 1 ml of PBS-washed Actigel resin (Sterogene) with region specific protein and 1/10 final volume of Ald-coupling solution (1M sodium cyanoborohydride). The total region specific protein added to each reaction mixture was 2.7 mg (interval 1), 3 mg (intervals 2 and 3), 0.1 mg (interval 4), 0.2 mg (interval 5) and 4 mg (interval 6). Protein for intervals 1, 3, and 6 was affinity purified pMAL fusion protein in column buffer (see Example 11). Interval 4 was affinity purified poly-histidine containing pET fusion in PBS; intervals 2 and 5 were from inclusion body preparations of insoluble pMAL fusion protein, dialyzed extensively in PBS. Aliquots of the supernatants from the coupling reactions, before and after coupling, were



assessed by Coomassie staining of 7.5% SDS-PAGE gels. Based on protein band intensities, in all cases greater than 50% coupling efficiencies were estimated. The resins were poured into 5 ml BioRad columns, washed extensively with PBS, and stored at 4°C.

5 Aliquots of the CTA IgY polyclonal antibody preparation were depleted for each individual region as described below. A 20 ml sample of the CTA IgY preparation [Example 8(c)] was dialyzed extensively against 3 changes of PBS (1 liter for each dialysis), quantitated by absorbance at OD<sub>280</sub>, and stored at 4°C. Six 1 ml aliquots of the dialyzed IgY preparation were removed, and depleted individually for each of the six intervals. Each 1 ml aliquot was passed over the appropriate affinity column, and the eluate twice reappplied to the column.  
10 The eluate was collected, and pooled with a 1 ml PBS wash. Bound antibody was eluted from the column by washing with 5 column volumes of 4 M Guanidine-HCl (in 10 mM Tris-HCl, pH 8.0). The column was reequilibrated in PBS, and the depleted antibody stock reappplied as described above. The eluate was collected, pooled with a 1 ml PBS wash, quantitated by absorbance at OD<sub>280</sub>, and stored at 4°C. In this manner, 6 aliquots of the CTA  
15 IgY preparation were individually depleted for each of the 6 toxin A intervals, by two rounds of affinity depletion. The specificity of each depleted stock was tested by Western blot analysis. Multiple 7.5% SDS-PAGE gels were loaded with protein samples corresponding to all 6 toxin A subregions. After electrophoresis, the gels were blotted, and protein transfer confirmed by Ponceau S staining [protocols described in Williams *et al.* (1995), *supra*]. After  
20 blocking the blots 1 hr at 20°C in PBS+ 0.1% Tween 20 (PBST) containing 5% milk (as a blocking buffer), 4 ml of either a 1/500 dilution of the dialyzed CTA IgY preparation in blocking buffer, or an equivalent amount of the six depleted antibody stocks (using OD<sub>280</sub> to standardize antibody concentration) were added and the blots incubated a further 1 hr at room temperature. The blots were washed and developed with alkaline phosphatase (using a rabbit  
25 anti-chicken alkaline phosphate conjugate as a secondary antibody) as previously described [Williams *et al.* (1995), *supra*]. In all cases, only the target interval was depleted for antibody reactivity, and at least 90% of the reactivity to the target intervals was specifically depleted.

Region specific antibody pools were isolated by affinity chromatography as described below. Ten mls of the dialyzed CTA IgY preparation were applied sequentially to each  
30 affinity column, such that a single 10 ml aliquot was used to isolate region specific antibodies specific to each of the six subregions. The columns were sequentially washed with 10 volumes of PBS, 6 volumes of BBS-Tween, 10 volumes of TBS, and eluted with 4 ml Actisep elution media (Sterogene). The eluate was dialyzed extensively against several

changes of PBS, and the affinity purified antibody collected and stored at 4°C. The volumes of the eluate increased to greater than 10 mls during dialysis in each case, due to the high viscosity of the Actisep elution media. Aliquots of each sample were 20x concentrated using Centricon 30 microconcentrators (Amicon) and stored at 4°C. The specificity of each region specific antibody pool was tested, relative to the dialyzed CTA IgY preparation, by Western blot analysis, exactly as described above, except that 4 ml samples of blocking buffer containing 100 µl region specific antibody (unconcentrated) were used instead of the depleted CTA IgY preparations. Each affinity purified antibody preparation was specific to the defined interval, except that samples purified against intervals 1-5 also reacted with interval 6. This may be due to non-specific binding to the interval 6 protein, since this protein contains the repetitive ligand binding domain which has been shown to bind antibodies nonspecifically. [Lyerty *et al.*, Curr. Microbiol., 19:303-306 (1989).]

The reactivity of each affinity purified antibody preparation to the corresponding proteins was approximately the same as the reactivity of the 1/500 diluted dialyzed CTA IgY preparation standard. Given that the specific antibody stocks were diluted 1/40, this would indicate that the unconcentrated affinity purified antibody stocks contain 1/10-1/20 the concentration of specific antibodies relative to the starting CTA IgY preparation.

**c) Toxin A Neutralization Assay Using Antibodies Reactive  
Toward Recombinant Toxin A Protein**

The C110 toxin neutralization assay [Example 8(d)] was used to assess the ability of the depleted or enriched samples generated in (b) above to neutralize the cytotoxicity of toxin A. The general ability of affinity purified antibodies to neutralize toxin A was assessed by mixing together aliquots of all 6 concentrated stocks of the 6 affinity purified samples generated in (b) above, and testing the ability of this mixture to neutralize a toxin A concentration of 0.1 µg/ml. The results, shown in Figure 11, demonstrate almost complete neutralization of toxin A using the affinity purified (AP) mix. Some epitopes within the recombinant proteins utilized for affinity purification were probably lost when the proteins were denatured before affinity purification [by Guanidine-HCl treatment in (b) above]. Thus, the neutralization ability of antibodies directed against recombinant protein is probably underestimated using these affinity purified antibody pools. This experiment demonstrates that antibodies reactive to recombinant toxin A can neutralize cytotoxicity, suggesting that

neutralizing antibodies may be generated by using recombinant toxin A protein as immunogen.

In view of the observation that the recombinant expression clones of the toxin A gene divide the protein into 6 subregions, the neutralizing ability of antibodies directed against each individual region was assessed. The neutralizing ability of antibodies directed against the ligand binding domain of toxin A was determined first.

In the toxin neutralization experiment shown in Figure 11, interval 6 specific antibodies (interval 6 contains the ligand binding domain) were depleted from the dialyzed PEG preparation, and the effect on toxin neutralization assayed. Interval 6 antibodies were depleted either by utilizing the interval 6 depleted CTA IgY preparation from (b) above ("6 aff. depleted" in Figure 11), or by addition of interval 6 protein to the CTA IgY preparation (estimated to be a 10 fold molar excess over anti-interval 6 immunoglobulin present in this preparation) to competitively compete for interval 6 protein ("6 prot depleted" in Figure 11). In both instances, removal of interval 6 specific antibodies reduces the neutralization efficiency relative to the starting CTA IgY preparation. This demonstrates that antibodies directed against interval 6 contribute to toxin neutralization. Since interval 6 corresponds to the ligand binding domain of the protein, these results demonstrate that antibodies directed against this region in the PEG preparation contribute to the neutralization of toxin A in this assay. However, it is significant that after removal of these antibodies, the PEG preparation retains significant ability to neutralize toxin A (Figure 11). This neutralization is probably due to the action of antibodies specific to other regions of the toxin A protein, since at least 90% of the ligand binding region reactive antibodies were removed in the depleted sample prepared in (b) above. This conclusion was supported by comparison of the toxin neutralization of the affinity purified (AP) mix compared to affinity purified interval 6 antibody alone. Although some neutralization ability was observed with AP interval 6 antibodies alone, the neutralization was significantly less than that observed with the mixture of all 6 AP antibody stocks (not shown).

Given that the mix of all six affinity purified samples almost completely neutralized the cytotoxicity of toxin A (Figure 11), the relative importance of antibodies directed against toxin A intervals 1-5 within the mixture was determined. This was assessed in two ways. First, samples containing affinity purified antibodies representing 5 of the 6 intervals were prepared, such that each individual region was depleted from one sample. Figure 12 demonstrates a sample neutralization curve, comparing the neutralization ability of affinity

purified antibody mixes without interval 4 (-4) or 5 (-5) specific antibodies, relative to the mix of all 6 affinity purified antibody stocks (positive control). While the removal of interval 5 specific antibodies had no effect on toxin neutralization (or intervals 1-3, not shown), the loss of interval 4 specific antibodies significantly reduced toxin neutralization (Figure 12).

Similar results were seen in a second experiment, in which affinity purified antibodies, directed against a single region, were added to interval 6 specific antibodies, and the effects on toxin neutralization assessed. Only interval 4 specific antibodies significantly enhanced neutralization when added to interval 6 specific antibodies (Figure 13). These results demonstrate that antibodies directed against interval 4 (corresponding to clone pPA1100-1450 in Figure 9) are important for neutralization of cytotoxicity in this assay. Epitope mapping has shown that only low levels of antibodies reactive to this region are generated when native toxin A is used as an immunogen [Example 12(a)]. It is hypothesized that immunization with recombinant protein specific to this interval will elicit higher titers of neutralizing antibodies. In summary, this analysis has identified two critical regions of the toxin A protein against which neutralizing antibodies are produced, as assayed by the CHO neutralization assay.

### EXAMPLE 13

#### Production And Evaluation Of Avian Antitoxin Against *C. difficile* Recombinant Toxin A Polypeptide

In Example 12, we demonstrated neutralization of toxin A mediated cytotoxicity by affinity purified antibodies reactive to recombinant toxin A protein. To determine whether antibodies raised against a recombinant polypeptide fragment of *C. difficile* toxin A may be effective in treating clostridial diseases, antibodies to recombinant toxin A protein representing the binding domain were generated. Two toxin A binding domain recombinant polypeptides, expressing the binding domain in either the pMALc (pMA1870-2680) or pET 23(pPA1870-2680) vector, were used as immunogens. The pMAL protein was affinity purified as a soluble product [Example 12(d)] and the pET protein was isolated as insoluble inclusion bodies [Example 12(d)] and solubilized to an immunologically active protein using a proprietary method described in a pending patent application (U.S. Patent Application Serial No. 08/129,027). This Example involves (a) immunization, (b) antitoxin collection, (c) determination of antitoxin antibody titer, (d) anti-recombinant toxin A neutralization of toxin A hemagglutination activity *in vitro*, and (e) assay of *in vitro* toxin A neutralizing activity.

**a) Immunization**

The soluble and the inclusion body preparations each were used separately to immunize hens. Both purified toxin A polypeptides were diluted in PBS and emulsified with approximately equal volumes of CFA for the initial immunization or IFA for subsequent booster immunizations. On day zero, for each of the recombinant preparations, two egg laying white Leghorn hens (obtained from local breeder) were each injected at multiple sites (intramuscular and subcutaneous) with 1 ml of recombinant adjuvant mixture containing approximately 0.5 to 1.5 mgs of recombinant toxin A. Booster immunizations of 1.0 mg were given on days 14 and day 28.

**b) Antitoxin Collection**

Total yolk immune IgY was extracted as described in the standard PEG protocol (as in Example 1) and the final IgY pellet was dissolved in sterile PBS at the original yolk volume. This material is designated "immune recombinant IgY" or "immune IgY."

**c) Antitoxin Antibody Titer**

To determine if the recombinant toxin A protein was sufficiently immunogenic to raise antibodies in hens, the antibody titer of a recombinant toxin A polypeptide was determined by ELISA. Eggs from both hens were collected on day 32, the yolks pooled and the antibody was isolated using PEG as described. The immune recombinant IgY antibody titer was determined for the soluble recombinant protein containing the maltose binding protein fusion generated in p-Mal (pMA1870-2680). Ninety-six well Falcon Pro-bind plates were coated overnight at 4°C with 100 µl /well of toxin A recombinant at 2.5 µg /µl in PBS containing 0.05% thimerosal. Another plate was also coated with maltose binding protein (MBP) at the same concentration, to permit comparison of antibody reactivity to the fusion partner. The next day, the wells were blocked with PBS containing 1% bovine serum albumin (BSA) for 1 hour at 37°C. IgY isolated from immune or preimmune eggs was diluted in antibody diluent (PBS containing 1% BSA and 0.05% Tween-20), and added to the blocked wells and incubated for 1 hour at 37°C. The plates were washed three times with PBS with 0.05% Tween-20, then three times with PBS. Alkaline phosphatase conjugated rabbit anti-chicken IgG (Sigma) diluted 1:1000 in antibody diluent was added to the plate, and incubated for 1 hour at 37°C. The plates were washed as before and substrate was added. [p-nitrophenyl phosphate (Sigma)] at 1 mg/ml in 0.05M Na<sub>2</sub>CO<sub>3</sub>, pH 9.5 and 10 mM MgCl<sub>2</sub>. The plates

were evaluated quantitatively on a Dynatech MR 300 Micro EPA plate reader at 410 nm about 10 minutes after the addition of substrate.

Based on these ELISA results, high antibody titers were raised in chickens immunized with the toxin A recombinant polypeptide. The recombinant appeared to be highly immunogenic, as it was able to generate high antibody titers relatively quickly with few immunizations. Immune IgY titer directed specifically to the toxin A portion of the recombinant was higher than the immune IgY titer to its fusion partner, the maltose binding protein, and significantly higher than the preimmune IgY. ELISA titers (reciprocal of the highest dilution of IgY generating a signal) in the preimmune IgY to the MBP or the recombinant was <1:30 while the immune IgY titers to MBP and the toxin A recombinant were 1:18750 and > 1:93750 respectively. Importantly, the anti-recombinant antibody titers generated in the hens against the recombinant polypeptide is much higher, compared to antibodies to that region raised using native toxin A. The recombinant antibody titer to region 1870-2680 in the CTA antibody preparation is at least five-fold lower compared to the recombinant generated antibodies (1:18750 versus >1:93750). Thus, it appears a better immune response can be generated against a specific recombinant using that recombinant as the immunogen compared to the native toxin A.

This observation is significant, as it shows that because recombinant portions stimulate the production of antibodies, it is not necessary to use native toxin molecules to produce antitoxin preparations. Thus, the problems associated with the toxicity of the native toxin are avoided and large-scale antitoxin production is facilitated.

#### **d) Anti-Recombinant Toxin A Neutralization Of Toxin A Hemagglutination Activity *In Vitro***

Toxin A has hemagglutinating activity besides cytotoxic and enterotoxin properties. Specifically, toxin A agglutinates rabbit erythrocytes by binding to a trisaccharide (gal 1-3B1-4GlcNAc) on the cell surface. [H. Krivan *et al.*, Infect. Immun., 53:573-581 (1986).] We examined whether the anti-recombinant toxin A (immune IgY, antibodies raised against the insoluble product expressed in pET) can neutralize the hemagglutination activity of toxin A in vitro. The hemagglutination assay procedure used was described by H.C. Krivan *et al.* Polyethylene glycol-fractionated immune or preimmune IgY were pre-absorbed with citrated rabbit erythrocytes prior to performing the hemagglutination assay because we have found that IgY alone can agglutinate red blood cells. Citrated rabbit red blood cells (RRBC's)(Cocalico)

were washed twice by centrifugation at 450 x g with isotonic buffer (0.1 M Tris-HCl, 0.05 M NaCl, pH 7.2). RRBC-reactive antibodies in the IgY were removed by preparing a 10% RRBC suspension (made by adding packed cells to immune or preimmune IgY) and incubating the mixture for 1 hour at 37°C. The RRBCs were then removed by centrifugation.

5 Neutralization of the hemagglutination activity of toxin A by antibody was tested in round-bottomed 96-well microtiter plates. Twenty-five µl of toxin A (36 µg/ml) (Tech Lab) in isotonic buffer was mixed with an equal volume of different dilutions of immune or preimmune IgY in isotonic buffer, and incubated for 15 minutes at room temperature. Then, 50 µl of a 1% RRBC suspension in isotonic buffer was added and the mixture was incubated

10 for 3 hours at 4°C. Positive control wells containing the final concentration of 9 µg/ml of toxin A after dilution without IgY were also included. Hemagglutination activity was assessed visually, with a diffuse matrix of RRBC's coating the bottom of the well representing a positive hemagglutination reaction and a tight button of RRBC's at the bottom of the well representing a negative reaction. The anti-recombinant immune IgY neutralized

15 toxin A hemagglutination activity, giving a neutralization titer of 1:8. However, preimmune IgY was unable to neutralize the hemagglutination ability of toxin A.

#### c) Assay Of *In Vitro* Toxin A Neutralizing Activity

The ability of the anti-recombinant toxin A IgY (immune IgY antibodies raised against pMA1870-2680, the soluble recombinant binding domain protein expressed in pMAL, designated as Anti-tox. A-2 in Figure 14, and referred to as recombinant region 6) and pre-immune IgY, prepared as described in Example 8(c) above, to neutralize the cytotoxic activity of toxin A was assessed *in vitro* using the CHO cell cytotoxicity assay, and toxin A (Tech Lab) at a concentration of 0.1 µg/ml, as described in Example 8(d) above. As additional

20 controls, the anti-native toxin A IgY (CTA) and pre-immune IgY preparations described in Example 8(c) above were also tested. The results are shown in Figure 14.

The anti-recombinant toxin A IgY demonstrated only partial neutralization of the cytotoxic activity of toxin A, while the pre-immune IgY did not demonstrate any significant neutralizing activity.

**EXAMPLE 14***In vivo* Neutralization Of *C. difficile* Toxin A

The ability of avian antibodies (IgY) raised against recombinant toxin A binding domain to neutralize the enterotoxin activity of *C. difficile* toxin A was evaluated *in vivo* using Golden Syrian hamsters. The Example involved: (a) preparation of the avian anti-recombinant toxin A IgY for oral administration; (b) *in vivo* protection of hamsters from *C. difficile* toxin A enterotoxicity by treatment of toxin A with avian anti-recombinant toxin A IgY; and (c) histologic evaluation of hamster ceca.

**a) Preparation Of The Avian Anti-Recombinant Toxin A IgY  
For Oral Administration**

Eggs were collected from hens which had been immunized with the recombinant *C. difficile* toxin A fragment pMA1870-2680 (described in Example 13, above). A second group of eggs purchased at a local supermarket was used as a pre-immune (negative) control. Egg yolk immunoglobulin (IgY) was extracted by PEG from the two groups of eggs as described in Example 8(c), and the final IgY pellets were solubilized in one-fourth the original yolk volume using 0.1M carbonate buffer (mixture of NaHCO<sub>3</sub> and Na<sub>2</sub>CO<sub>3</sub>), pH 9.5. The basic carbonate buffer was used in order to protect the toxin A from the acidic pH of the stomach environment.

**b) *In vivo* Protection Of Hamsters Against *C. difficile* Toxin A  
Enterotoxicity By Treatment Of Toxin A With Avian Anti-  
recombinant Toxin A IgY**

In order to assess the ability of the avian anti-recombinant toxin A IgY, prepared in section (a) above to neutralize the *in vivo* enterotoxin activity of toxin A, an *in vivo* toxin neutralization model was developed using Golden Syrian hamsters. This model was based on published values for the minimum amount of toxin A required to elicit diarrhea (0.08 mg toxin A/Kg body wt.) and death (0.16 mg toxin A/Kg body wt.) in hamsters when administered orally (Lyerly *et al.* Infect. Immun., 47:349-352 (1985).

For the study, four separate experimental groups were used, with each group consisting of 7 female Golden Syrian hamsters (Charles River), approx. three and one-half weeks old.



weighing approx. 50 gms each. The animals were housed as groups of 3 and 4, and were offered food and water *ad libitum* through the entire length of the study.

For each animal, a mixture containing either 10µg of toxin A (0.2 mg/Kg) or 30µg of toxin A (0.6 mg/Kg) (*C. difficile* toxin A was obtained from Tech Lab and 1 ml of either the anti-recombinant toxin A IgY or pre-immune IgY (from section (a) above) was prepared. These mixtures were incubated at 37°C for 60 min. and were then administered to the animals by the oral route. The animals were then observed for the onset of diarrhea and death for a period of 24 hrs. following the administration of the toxin A+IgY mixtures. at the end of which time, the following results were tabulated and shown in Table 17:

TABLE 17

Study Outcome At 24 Hours

Experimental Group	Study Outcome at 24 Hours		
	Healthy <sup>1</sup>	Diarrhea <sup>2</sup>	Dead <sup>3</sup>
10 µg Toxin A - Antitoxin Against Interval 6	7	0	0
30 µg Toxin A - Antitoxin Against Interval 6	7	0	0
10 µg Toxin A - Pre-Immune Serum	0	5	2
30 µg Toxin A - Pre-Immune	0	5	2

<sup>1</sup> Animals remained healthy through the entire 24 hour study period.

<sup>2</sup> Animals developed diarrhea, but did not die.

<sup>3</sup> Animals developed diarrhea, and subsequently died.

Pretreatment of toxin A at both doses tested, using the anti-recombinant toxin A IgY, prevented all overt symptoms of disease in hamsters. Therefore, pretreatment of *C. difficile* toxin A, using the anti-recombinant toxin A IgY, neutralized the *in vivo* enterotoxin activity of the toxin A. In contrast, all animals from the two groups which received toxin A which had been pretreated using pre-immune IgY developed disease symptoms which ranged from diarrhea to death. The diarrhea which developed in the 5 animals which did not die in each of the two pre-immune groups, spontaneously resolved by the end of the 24 hr. study period.

### c) Histologic Evaluation Of Hamster Cecca

In order to further assess the ability of anti-recombinant toxin A IgY to protect hamsters from the enterotoxin activity of toxin A, histologic evaluations were performed on the ceca of hamsters from the study described in section (b) above.

Three groups of animals were sacrificed in order to prepare histological specimens. The first group consisted of a single representative animal taken from each of the 4 groups of

surviving hamsters at the conclusion of the study described in section (b) above. These animals represented the 24 hr. timepoint of the study.

The second group consisted of two animals which were not part of the study described above, but were separately treated with the same toxin A + pre-immune IgY mixtures as described for the animals in section (b) above. Both of these hamsters developed diarrhea, and were sacrificed 8 hrs. after the time of administration of the toxin A + pre-immune IgY mixtures. At the time of sacrifice, both animals were presenting symptoms of diarrhea. These animals represented the acute phase of the study.

The final group consisted of a single untreated hamster from the same shipment of animals as those used for the two previous groups. This animal served as the normal control.

Samples of cecal tissue were removed from the 7 animals described above, and were fixed overnight at 4°C using 10% buffered formalin. The fixed tissues were paraffin-embedded, sectioned, and mounted on glass microscope slides. The tissue sections were then stained using hematoxylin and eosin (H and E stain), and were examined by light microscopy.

The tissues obtained from the two 24 hr. animals which received mixtures containing either 10µg or 30µg of toxin A and anti-recombinant toxin A IgY were indistinguishable from the normal control, both in terms of gross pathology, as well as at the microscopic level. These observations provide further evidence for the ability of anti-recombinant toxin A IgY to effectively neutralize the *in vivo* enterotoxin activity of *C. difficile* toxin A, and thus its ability to prevent acute or lasting toxin A-induced pathology.

In contrast, the tissues from the two 24 hr. animals which received the toxin A + pre-immune IgY mixtures demonstrated significant pathology. In both of these groups, the mucosal layer was observed to be less organized than in the normal control tissue. The cytoplasm of the epithelial cells had a vacuolated appearance, and gaps were present between the epithelium and the underlying cell layers. The lamina propria was largely absent. Intestinal villi and crypts were significantly diminished, and appeared to have been overgrown by a planar layer of epithelial cells and fibroblasts. Therefore, although these animals overtly appeared to recover from the acute symptoms of toxin A intoxication, lasting pathologic alterations to the cecal mucosa had occurred.

The tissues obtained from the two acute animals which received mixtures of toxin A and pre-immune IgY demonstrated the most significant pathology. At the gross pathological level, both animals were observed to have severely distended ceca which were filled with watery, diarrhea-like material. At the microscopic level, the animal that was given the

mixture containing 10µg of toxin A and pre-immune IgY was found to have a mucosal layer which had a ragged, damaged appearance, and a disorganized, compacted quality. The crypts were largely absent, and numerous breaks in the epithelium had occurred. There was also an influx of erythrocytes into spaces between the epithelial layer and the underlying tissue. The animal which had received the mixture containing 30µg of toxin A and pre-immune IgY demonstrated the most severe pathology. The cecal tissue of this animal had an appearance very similar to that observed in animals which had died from *C. difficile* disease. Widespread destruction of the mucosa was noted, and the epithelial layer had sloughed. Hemorrhagic areas containing large numbers of erythrocytes were very prevalent. All semblance of normal tissue architecture was absent from this specimen. In terms of the presentation of pathologic events, this *in vivo* hamster model of toxin A-intoxication correlates very closely with the pathologic consequences of *C. difficile* disease in hamsters. The results presented in this Example demonstrate that while anti-recombinant toxin A (Interval 6) IgY is capable of only partially neutralizing the cytotoxic activity of *C. difficile* toxin A, the same antibody effectively neutralizes 100% of the *in vivo* enterotoxin activity of the toxin. While it is not intended that this invention be limited to this mechanism, this may be due to the cytotoxicity and enterotoxicity of *C. difficile* Toxin A as two separate and distinct biological functions.

#### EXAMPLE 15

##### *In Vivo* Neutralization Of *C. Difficile* Toxin A By Antibodies Against Recombinant Toxin A Polypeptides

The ability of avian antibodies directed against the recombinant *C. difficile* toxin A fragment 1870-2680 (as expressed by pMA1870-2680; see Example 13) to neutralize the enterotoxic activity of toxin A was demonstrated in Example 14. The ability of avian antibodies (IgYs) directed against other recombinant toxin A epitopes to neutralize native toxin A *in vivo* was next evaluated. This example involved: (a) the preparation of IgYs against recombinant toxin A polypeptides; (b) *in vivo* protection of hamsters against toxin A by treatment with anti-recombinant toxin A IgYs and (c) quantification of specific antibody concentration in CTA and Interval 6 IgY PEG preparations.

The nucleotide sequence of the coding region of the entire toxin A protein is listed in SEQ ID NO:5. The amino acid sequence of the entire toxin A protein is listed in SEQ ID NO:6. The amino acid sequence consisting of amino acid residues 1870 through 2680 of

toxin A is listed in SEQ ID NO:7. The amino acid sequence consisting of amino acid residues 1870 through 1960 of toxin A is listed in SEQ ID NO:8.

a) **Preparation Of IgY's Against Recombinant Toxin A Polypeptides**

Eggs were collected from Leghorn hens which have been immunized with recombinant *C. difficile* toxin A polypeptide fragments encompassing the entire toxin A protein. The polypeptide fragments used as immunogens were: 1) pMA 1870-2680 (Interval 6), 2) pPA 1100-1450 (Interval 4), and 3) a mixture of fragments consisting of pMA 30-300 (Interval 1), pMA 300-660 (Interval 2), pMA 660-1100 (Interval 3) and pMA 1450-1870 (Interval 5). This mixture of immunogens is referred to as Interval 1235. The location of each interval within the toxin A molecule is shown in Figure 15A. In Figure 15A, the following abbreviations are used: pP refers to the pET23 vector (New England BioLabs); pM refers to the pMAL<sup>TM</sup>-c vector (New England BioLabs); A refers to toxin A; the numbers refer to the amino acid interval expressed in the clone. (For example, the designation pMA30-300 indicates that the recombinant clone encodes amino acids 30-300 of toxin A and the vector used was pMAL<sup>TM</sup>-c).

The recombinant proteins were generated as described in Example 11. The IgYs were extracted and solubilized in 0.1M carbonate buffer pH 9.5 for oral administration as described in Example 14(a). The IgY reactivities against each individual recombinant interval was evaluated by ELISA as described in Example 13(c).

b) **In Vivo Protection Of Hamsters Against Toxin**

**A By Treatment With Anti-Recombinant Toxin A Antibodies**

The ability of antibodies raised against recombinant toxin A polypeptides to provide *in vivo* protection against the enterotoxic activity of toxin A was examined in the hamster model system. This assay was performed as described in Example 14(b). Briefly, for each 40-50 gram female Golden Syrian hamster (Charles River), 1 ml of IgY 4X (*i.e.*, resuspended in 1/4 of the original yolk volume) PEG prep against Interval 6, Interval 4 or Interval 1235 was mixed with 30 µg (LD<sub>50</sub> oral dose) of *C. difficile* toxin A (Tech Lab). Preimmune IgY mixed with toxin A served as a negative control. Antibodies raised against *C. difficile* toxoid A (Example 8) mixed with toxin A (CTA) served as a positive control. The mixture was incubated for 1 hour at 37°C then orally administered to lightly etherized hamsters using an

18G feeding needle. The animals were then observed for the onset of diarrhea and death for a period of approximately 24 hours. The results are shown in Table 18.

TABLE 18

Study Outcome After 24 Hours

Treatment group	Healthy <sup>1</sup>	Diarrhea <sup>2</sup>	Dead <sup>3</sup>
Preimmune	0	0	7
CTA	5	0	0
Interval 6	6	1	0
Interval 4	0	1	6
Interval 1235	0	0	7

<sup>1</sup> Animal shows no sign of illness.

<sup>2</sup> Animal developed diarrhea, but did not die.

<sup>3</sup> Animal developed diarrhea and died.

Pre-treatment of toxin A with IgYs against Interval 6 prevented diarrhea in 6 of 7 hamsters and completely prevented death in all 7. In contrast, as with preimmune IgY, IgYs against Interval 4 and Interval 1235 had no effect on the onset of diarrhea and death in the hamsters.

#### c) Quantification Of Specific Antibody Concentration In CTA And Interval 6 IgY PEG Preparations

To determine the purity of IgY PEG preparations, an aliquot of a pMA1870-2680 (Interval 6) IgY PEG preparation was chromatographed using HPLC and a KW-803 sizing column (Shodex). The resulting profile of absorbance at 280 nm is shown in Figure 16. The single large peak corresponds to the predicted MW of IgY. Integration of the area under the single large peak showed that greater than 95% of the protein eluted from the column was present in this single peak. This result demonstrated that the majority (>95%) of the material absorbing at 280 nm in the PEG preparation corresponds to IgY. Therefore, absorbance at 280 nm can be used to determine the total antibody concentration in PEG preparations.

To determine the concentration of Interval 6-specific antibodies (expressed as percent of total antibody) within the CTA and pMA1870-2680 (Interval 6) PEG preparations, defined quantities of these antibody preparations were affinity purified on a pPA1870-2680(II) (shown schematically in Figure 15B) affinity column and the specific antibodies were quantified. In Figure 15B the following abbreviations are used: pP refers to the pET23 vector (New England Biolabs); pM refers to the pMAL<sup>TM</sup>-c vector (New England Biolabs); pG refers to the pGEX

vector (Pharmacia); pB refers to the PinPoint™ Xa vector (Promega); A refers to toxin A; the numbers refer to the amino acid interval expressed in the clone. The solid black ovals represent the MBP; the hatched ovals represent glutathione S-transferase; the hatched circles represent the biotin tag; and HHHH represents the poly-histidine tag.

5        An affinity column containing recombinant toxin A repeat protein was made as follows. Four ml of PBS-washed Actigel resin (Sterogene) was coupled with 5-10 mg of pPA1870-2680 inclusion body protein [prepared as described in Example (17) and dialyzed into PBS] in a 15 ml tube (Falcon) containing 1/10 final volume Ald-coupling solution (1 M sodium cyanoborohydride). Aliquots of the supernatant from the coupling reactions, before  
10       and after coupling, were assessed by Coomassie staining of 7.5% SDS-PAGE gels. Based upon protein band intensities, greater than 6 mg of recombinant protein was coupled to the resin. The resin was poured into a 10 ml column (BioRad), washed extensively with PBS, pre-eluted with 4 M guanidine-HCl (in 10 mM Tris-HCl, pH 8.0; 0.005% thimerosal) and re-equilibrated with PBS. The column was stored at 4°C.

15       Aliquots of a pMA1870-2680 (Interval 6) or a CTA IgY polyclonal antibody preparation (PEG prep) were affinity purified on the above affinity column as follows. The column was attached to an UV monitor (ISCO) and washed with PBS. For pMA1870-2680 IgY purification, a 2X PEG prep (filter sterilized using a 0.45 µ filter; approximately 500 mg total IgY) was applied. The column was washed with PBS until the baseline was re-  
20       established (the column flow-through was saved), washed with BBSTween to elute nonspecifically binding antibodies and re-equilibrated with PBS. Bound antibody was eluted from the column in 4 M guanidine-HCl (in 10 mM Tris-HCl, pH 8.0; 0.005% thimerosal). The entire elution peak was collected in a 15 ml tube (Falcon). The column was re-equilibrated and the column eluate was re-chromatographed as described above. The antibody  
25       preparation was quantified by UV absorbance (the elution buffer was used to zero the spectrophotometer). Total purified antibody was approximately 9 mg and 1 mg from the first and second chromatography passes, respectively. The low yield from the second pass indicated that most specific antibodies were removed by the first round of chromatography. The estimated percentage of Interval 6 specific antibodies in the pMA1870-2680 PEG prep is  
30       approximately 2%.

The percentage of Interval 6 specific antibodies in the CTA PEG prep was determined (utilizing the same column and methodology described above) to be approximately 0.5% of total IgY.

A 4X PEG prep contains approximately 20 mg/ml IgY. Thus in b) above, approximately 400 µg specific antibody in the Interval 6 PEG prep neutralized 30 µg toxin A *in vivo*.

## EXAMPLE 16

### *In Vivo* Treatment Of *C. difficile* Disease In Hamsters By Recombinant Interval 6 Antibodies

The ability of antibodies directed against recombinant Interval 6 of toxin A to protect hamsters *in vivo* from *C. difficile* disease was examined. This example involved: (a) prophylactic treatment of *C. difficile* disease and (b) therapeutic treatment of *C. difficile* disease.

#### a) Prophylactic Treatment Of *C. difficile* Disease

This experiment was performed as described in Example 9(b). Three groups each consisting of 7 female 100 gram Syrian hamsters (Charles River) were prophylactically treated with either preimmune IgYs, IgYs against native toxin A and B [CTAB: see Example 8 (a) and (b)] or IgYs against Interval 6. IgYs were prepared as 4X PEG preparations as described in Example 9(a).

The animals were orally dosed 3 times daily, roughly at 4 hour intervals, for 12 days with 1 ml antibody preparations diluted in Ensure®. Using estimates of specific antibody concentration from Example 15(c), each dose of the Interval 6 antibody prep contained approximately 400 µg of specific antibody. On day 2 each hamster was predisposed to *C. difficile* infection by the oral administration of 3.0 mg of Clindamycin-HCl (Sigma) in 1 ml of water. On day 3 the hamsters were orally challenged with 1 ml of *C. difficile* inoculum strain ATCC 43596 in sterile saline containing approximately 100 organisms. The animals were then observed for the onset of diarrhea and subsequent death during the treatment period. The results are shown in Table 19.

TABLE 19

Lethality After 12 Days Of Treatment

Treatment Group	Number Animals Alive	Number Animals Dead
Preimmune	0	7
CTAB	6	1
Interval 6	7	0

Treatment of hamsters with orally-administered IgYs against Interval 6 successfully protected 7 out of 7 (100%) of the animals from *C. difficile* disease. One of the hamsters in this group presented with diarrhea which subsequently resolved during the course of treatment. As shown previously in Example 9, antibodies to native toxin A and toxin B were highly protective. In this Example, 6 out of 7 animals survived in the CTAB treatment group. All of the hamsters treated with preimmune sera came down with diarrhea and died. The survivors in both the CTAB and Interval 6 groups remained healthy throughout a 12 day post-treatment period. In particular, 6 out of 7 Interval 6-treated hamsters survived at least 2 weeks after termination of treatment which suggests that these antibodies provide a long-lasting cure. These results represent the first demonstration that antibodies generated against a recombinant region of toxin A can prevent CDAD when administered passively to animals. These results also indicate that antibodies raised against Interval 6 alone may be sufficient to protect animals from *C. difficile* disease when administered prophylactically.

Previously others had raised antibodies against toxin A by actively immunizing hamsters against a recombinant polypeptide located within the Interval 6 region [Lyerly, D.M., *et al.* (1990) *Curr. Microbiol.* 21:29]. Figure 17 shows schematically the location of the Lyerly, *et al.* intra-Interval 6 recombinant protein (cloned into the pUC vector) in comparison with the complete Interval 6 construct (pMA1870-2680) used herein to generate neutralizing antibodies directed against toxin A. In Figure 17, the solid black oval represents the MBP which is fused to the toxin A Interval 6 in pMA1870-2680.

The Lyerly, *et al.* antibodies (intra-Interval 6) were only able to partially protect hamsters against *C. difficile* infection in terms of survival (4 out of 8 animals survived) and furthermore, these antibodies did not prevent diarrhea in any of the animals. Additionally, animals treated with the intra-Interval 6 antibodies [Lyerly, *et al.* (1990), *supra*] died when treatment was removed.

In contrast, the experiment shown above demonstrates that passive administration of anti-Interval 6 antibodies prevented diarrhea in 6 out of 7 animals and completely prevented



death due to CDAD. Furthermore, as discussed above, passive administration of the anti-Interval 6 antibodies provides a long lasting cure (*i.e.*, treatment could be withdrawn without incident).

5                   b)     **Therapeutic Treatment Of *C. difficile* Disease: *In Vivo***  
                    **Treatment Of An Established *C. difficile* Infection In**  
                    **Hamsters With Recombinant Interval 6 Antibodies**

10                   The ability of antibodies against recombinant interval 6 of toxin A to therapeutically treat *C. difficile* disease was examined. The experiment was performed essentially as described in Example 10(b). Three groups, each containing seven to eight female Golden Syrian hamsters (100 g each; Charles River) were treated with either preimmune IgY, IgYs against native toxin A and toxin B (CTAB) and IgYs against Interval 6. The antibodies were prepared as described above as 4X PEG preparations.

15                   The hamsters were first predisposed to *C. difficile* infection with a 3 mg dose of Clindamycin-HCl (Sigma) administered orally in 1 ml of water. Approximately 24 hrs later, the animals were orally challenged with 1 ml of *C. difficile* strain ATCC 43596 in sterile saline containing approximately 200 organisms. One day after infection, the presence of toxin A and B was determined in the feces of the hamsters using a commercial immunoassay kit (Cytoclone A+B EPA, Cambridge Biotech) to verify establishment of infection. Four  
20                   members of each group were randomly selected and tested. Feces from an uninfected hamster was tested as a negative control. All infected animals tested positive for the presence of toxin according to the manufacturer's procedure. The initiation of treatment then started approximately 24 hr post-infection.

25                   The animals were dosed daily at roughly 4 hr intervals with 1 ml antibody preparation diluted in Ensure® (Ross Labs). The amount of specific antibodies given per dose (determined by affinity purification) was estimated to be about 400 µg of anti-Interval 6 IgY (for animals in the Interval 6 group) and 100 µg and 70 µg of anti-toxin A (Interval 6-specific) and anti-toxin B (Interval 3-specific; see Example 19), respectively, for the CTAB preparation. The animals were treated for 9 days and then observed for an additional 4 days  
30                   for the presence of diarrhea and death. The results indicating the number of survivors and the number of dead 4 days post-infection are shown in Table 20.

TABLE 20

*In vivo* Therapeutic Treatment With Interval 6 Antibodies

Treatment Group	Number Animals Alive	Number Animals Dead
Preimmune	4	3
CTAB	8	0
Interval 6	8	0

Antibodies directed against both Interval 6 and CTAB successfully prevented death from *C. difficile* when therapeutically administered 24 hr after infection. This result is significant since many investigators begin therapeutic treatment of hamsters with existing drugs (e.g., vancomycin, phenelfamycins, ticamycins, etc.) 8 hr post-infection [Swanson, *et al.* (1991) Antimicrobial Agents and Chemotherapy 35:1108 and (1989) J. Antibiotics 42:94].

Forty-two percent of hamsters treated with preimmune IgY died from CDAD. While the anti-Interval 6 antibodies prevented death in the treated hamsters, they did not eliminate all symptoms of CDAD as 3 animals presented with slight diarrhea. In addition, one CTAB-treated and one preimmune-treated animal also had diarrhea 14 days post-infection. These results indicate that anti-Interval 6 antibodies provide an effective means of therapy for CDAD.

### EXAMPLE 17

#### Induction Of Toxin A Neutralizing Antibodies Requires Soluble Interval 6 Protein

As shown in Examples 11(d) and 15, expression of recombinant proteins in *E. coli* may result in the production of either soluble or insoluble protein. If insoluble protein is produced, the recombinant protein is solubilized prior to immunization of animals. To determine whether, one or both of the soluble or insoluble recombinant proteins could be used to generate neutralizing antibodies to toxin A, the following experiment was performed. This example involved a) expression of the toxin A repeats and subfragments of these repeats in *E. coli* using a variety of expression vectors; b) identification of recombinant toxin A repeats and sub-regions to which neutralizing antibodies bind; and c) determination of the neutralization ability of antibodies raised against soluble and insoluble toxin A repeat immunogen.

a) **Expression Of The Toxin A Repeats And Subfragments Of These Repeats In *E. coli* Using A Variety Of Expression Vectors**

The Interval 6 immunogen utilized in Examples 15 and 16 was the pMA1870-2680 protein, in which the toxin A repeats are expressed as a soluble fusion protein with the MBP (described in Example 11). Interestingly, expression of this region (from the *SpeI* site to the end of the repeats, see Figure 15B) in three other expression constructs, as either native (pPA1870-2680), poly-His tagged [pPA1870-2680 (H)] or biotin-tagged (pBA1870-2680) proteins resulted in completely insoluble protein upon induction of the bacterial host (see Figure 15B). The host strain BL21 (Novagen) was used for expression of pBA1870-2680 and host strain BL21(DE3) (Novagen) was used for expression of pPA1870-2680 and pPA1870-2680(H). These insoluble proteins accumulated to high levels in inclusion bodies. Expression of recombinant plasmids in *E. coli* host cells grown in 2X YT medium was performed as described [Williams, *et al.* (1995), *supra*].

As summarized in Figure 15B, expression of fragments of the toxin A repeats (as either N-terminal *SpeI*-*EcoRI* fragments, or C-terminal *EcoRI*-end fragments) also yielded high levels of insoluble protein using pGEX (pGA1870-2190), PinPoint<sup>TM</sup>-Xa (pBA1870-2190 and pBA2250-2680) and pET expression systems (pPA1870-2190). The pGEX and pET expression systems are described in Example 11. The PinPoint<sup>TM</sup>-Xa expression system drives the expression of fusion proteins in *E. coli*. Fusion proteins from PinPoint<sup>TM</sup>-Xa vectors contain a biotin tag at the amino-terminal end and can be affinity purified SoftLink<sup>TM</sup> Soft Release avidin resin (Promega) under mild denaturing conditions (5 mM biotin).

The solubility of expressed proteins from the pGA1870-2190 and pPA1870-2190 expression constructs was determined after induction of recombinant protein expression under conditions reported to enhance protein solubility [These conditions comprise growth of the host at reduced temperature (30°C) and the utilization of high (1 mM IPTG) or low (0.1 mM IPTG) concentrations of inducer [Williams *et al.* (1995), *supra*]. All expressed recombinant toxin A protein was insoluble under these conditions. Thus, expression of these fragments of the toxin A repeats in pET and pGEX expression vectors results in the production of insoluble recombinant protein even when the host cells are grown at reduced temperature and using lower concentrations of the inducer. Although expression of these fragments in pMal vectors yielded affinity purifiable soluble fusion protein, the protein was either predominantly insoluble (pMA1870-2190) or unstable (pMA2250-2650). Attempts to solubilize expressed

protein from the pMA1870-2190 expression construct using reduced temperature or lower inducer concentration (as described above) did not improve fusion protein solubility.

Collectively, these results demonstrate that expression of the toxin A repeat region in *E. coli* results in the production of insoluble recombinant protein, when expressed as either large (aa 1870-2680) or small (aa 1870-2190 or aa 2250-2680) fragments, in a variety of expression vectors (native or poly-his tagged pET, pGEX or PinPoint<sup>TM</sup>- Xa vectors), utilizing growth conditions shown to enhance protein solubility. The exception to this rule were fusions with the MBP, which enhanced protein solubility, either partially (pMA1870-2190) or fully (pMA1870-2680).

#### b) Identification Of Recombinant Toxin A Repeats And Sub-Regions To Which Neutralizing Antibodies Bind

Toxin A repeat regions to which neutralizing antibodies bind were identified by utilizing recombinant toxin A repeat region proteins expressed as soluble or insoluble proteins to deplete protective antibodies from a polyclonal pool of antibodies against native *C. difficile* toxin A. An *in vivo* assay was developed to evaluate proteins for the ability to bind neutralizing antibodies.

The rationale for this assay is as follows. Recombinant proteins were first pre-mixed with antibodies against native toxin A (CTA antibody: generated in Example 8) and allowed to react. Subsequently, *C. difficile* toxin A was added at a concentration lethal to hamsters and the mixture was administered to hamsters via IP injection. If the recombinant protein contains neutralizing epitopes, the CTA antibodies would lose their ability to bind toxin A resulting in diarrhea and/or death of the hamsters.

The assay was performed as follows. The lethal dose of toxin A when delivered orally to nine 40 to 50 g Golden Syrian hamsters (Sasco) was determined to be 10 to 30 µg. The PEG-purified CTA antibody preparation was diluted to 0.5X concentration (*i.e.*, the antibodies were diluted at twice the original yolk volume) in 0.1 M carbonate buffer, pH 9.5. The antibodies were diluted in carbonate buffer to protect them from acid degradation in the stomach. The concentration of 0.5X was used because it was found to be the lowest effective concentration against toxin A. The concentration of Interval 6-specific antibodies in the 0.5X CTA prep was estimated to be 10-15 µg/ml (estimated using the method described in Example 15).

The inclusion body preparation [insoluble Interval 6 protein: pPA1870-2680(H)] and the soluble Interval 6 protein [pMA1870-2680; see Figure 15] were both compared for their ability to bind to neutralizing antibodies against *C. difficile* toxin A (CTA). Specifically, 1 to 2 mg of recombinant protein was mixed with 5 ml of a 0.5X CTA antibody prep (estimated to contain 60-70 µg of Interval 6-specific antibody). After incubation for 1 hr at 37°C, CTA (Tech Lab) at a final concentration of 30 µg/ml was added and incubated for another 1 hr at 37°C. One ml of this mixture containing 30 µg of toxin A (and 10-15 µg of Interval 6-specific antibody) was administered orally to 40-50 g Golden Syrian hamsters (Sasco). Recombinant proteins that result in the loss of neutralizing capacity of the CTA antibody would indicate that those proteins contain neutralizing epitopes. Preimmune and CTA antibodies (both at 0.5X) without the addition of any recombinant protein served as negative and positive controls, respectively.

Two other inclusion body preparations, both expressed as insoluble products in the pET vector, were tested: one containing a different insert (toxin B fragment) other than Interval 6 called pPB1850-2070 (see Figure 18) which serves as a control for insoluble Interval 6, the other was a truncated version of the Interval 6 region called pPA1870-2190 (see Figure 15B). The results of this experiment are shown in Table 21.

TABLE 21

Binding Of Neutralizing Antibodies By Soluble Interval 6 Protein Study Outcome After 24 Hours

Treatment Group <sup>1</sup>	Healthy <sup>2</sup>	Diarrhea <sup>3</sup>	Dead <sup>4</sup>
Preimmune Ab	0	3	2
CTA Ab	4	1	0
CTA Ab + Int 6 (soluble)	1	2	2
CTA Ab + Int 6 (insoluble)	5	0	0
CTA Ab + pPB1850-2070	5	0	0
CTA Ab + pPA1870-2190	5	0	0

<sup>1</sup> *C. difficile* toxin A (CTA) was added to each group.

<sup>2</sup> Animals showed no signs of illness.

<sup>3</sup> Animals developed diarrhea but did not die.

<sup>4</sup> Animals developed diarrhea and died.

Preimmune antibody was ineffective against toxin A, while anti-CTA antibodies at a dilute 0.5X concentration almost completely protected the hamsters against the enterotoxic effects of CTA. The addition of recombinant proteins pPB1850-2070 or pPA1870-2190 to the anti-CTA antibody had no effect upon its protective ability, indicating that these recombinant proteins do not bind to neutralizing antibodies. On the other hand, recombinant

Interval 6 protein was able to bind to neutralizing anti-CTA antibodies and neutralized the *in vivo* protective effect of the anti-CTA antibodies. Four out of five animals in the group treated with anti-CTA antibodies mixed with soluble Interval 6 protein exhibited toxin associated toxicity (diarrhea and death). Moreover, the results showed that Interval 6 protein must be expressed as a soluble product in order for it to bind to neutralizing anti-CTA antibodies since the addition of insoluble Interval 6 protein had no effect on the neutralizing capacity of the CTA antibody prep.

**c) Determination Of Neutralization Ability Of Antibodies  
Raised Against Soluble And Insoluble Toxin A Repeat  
Immunogen**

To determine if neutralizing antibodies are induced against solubilized inclusion bodies, insoluble toxin A repeat protein was solubilized and specific antibodies were raised in chickens. Insoluble pPA1870-2680 protein was solubilized using the method described in Williams *et al.* (1995), *supra*. Briefly, induced cultures (500 ml) were pelleted by centrifugation at 3,000 X g for 10 min at 4°C. The cell pellets were resuspended thoroughly in 10 ml of inclusion body sonication buffer (25 mM HEPES pH 7.7, 100 mM KCl, 12.5 mM MgCl<sub>2</sub>, 20% glycerol, 0.1% (v/v) Nonidet P-40, 1 mM DTT). The suspension was transferred to a 30 ml non-glass centrifuge tube. Five hundred µl of 10 mg/ml lysozyme was added and the tubes were incubated on ice for 30 min. The suspension was then frozen at -70°C for at least 1 hr. The suspension was thawed rapidly in a water bath at room temperature and then placed on ice. The suspension was then sonicated using at least eight 15 sec bursts of the microprobe (Branson Sonicator Model No. 450) with intermittent cooling on ice.

The sonicated suspension was transferred to a 35 ml Oakridge tube and centrifuged at 6,000 X g for 10 min at 4°C to pellet the inclusion bodies. The pellet was washed 2 times by pipetting or vortexing in fresh, ice-cold RIPA buffer [0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate in TBS (25 mM Tris-Cl pH 7.5, 150 mM NaCl)]. The inclusion bodies were recentrifuged after each wash. The inclusion bodies were dried and transferred using a small metal spatula to a 15 ml tube (Falcon). One ml of 10% SDS was added and the pellet was solubilized by gently pipetting the solution up and down using a 1 ml micropipettor. The solubilization was facilitated by heating the sample to 95°C when necessary.

Once the inclusion bodies were in solution, the samples were diluted with 9 volumes of PBS. The protein solutions were dialyzed overnight against a 100-fold volume of PBS

containing 0.05% SDS at room temperature. The dialysis buffer was then changed to PBS containing 0.01% SDS and the samples were dialyzed for several hours to overnight at room temperature. The samples were stored at 4°C until used. Prior to further use, the samples were warmed to room temperature to allow any precipitated SDS to go back into solution.

The inclusion body preparation was used to immunize hens. The protein was dialyzed into PBS and emulsified with approximately equal volumes of CFA for the initial immunization or IFA for subsequent booster immunizations. On day zero, for each of the recombinant recombinant preparations, two egg laying white Leghorn hens were each injected at multiple sites (IM and SC) with 1 ml of recombinant protein-adjuvant mixture containing approximately 0.5-1.5 mg of recombinant protein. Booster immunizations of 1.0 mg were given on days 14 and day 28. Eggs were collected on day 32 and the antibody isolated using PEG as described in Example 14(a). High titers of toxin A specific antibodies were present (as assayed by ELISA, using the method described in Example 13). Titers were determined for both antibodies against recombinant polypeptides pPA1870-2680 and pMA1870-2680 and were found to be comparable at > 1:62,500.

Antibodies against soluble Interval 6 (pMA1870-2680) and insoluble Interval 6 [(inclusion body), pPA1870-2680] were tested for neutralizing ability against toxin A using the *in vivo* assay described in Example 15(b). Preimmune antibodies and antibodies against toxin A (CTA) served as negative and positive controls, respectively. The results are shown in Table 22.

TABLE 22

Neutralization Of Toxin A By Antibodies Against Soluble Interval 6 Protein Study Outcome After 24 Hours

Antibody Treatment Group	Healthy <sup>1</sup>	Diarrhea <sup>2</sup>	Dead <sup>3</sup>
Preimmune	1	0	4
CTA	5	0	0
Interval 6 (Soluble) <sup>4</sup>	5	0	0
Interval 6 (Insoluble)	0	2	3

<sup>1</sup> Animals showed no sign of illness.

<sup>2</sup> Animal developed diarrhea but did not die.

<sup>3</sup> Animal developed diarrhea and died.

<sup>4</sup> 400 µg/ml.

Antibodies raised against native toxin A were protective while preimmune antibodies had little effect. As found using the *in vitro* CHO assay [described in Example 8(d)] where antibodies raised against the soluble Interval 6 could partially neutralize the effects of toxin A, here they were able to completely neutralize toxin A *in vivo*. In contrast, the antibodies

raised against the insoluble Interval 6 was unable to neutralize the effects of toxin A *in vivo* as shown above (Table 22) and *in vitro* as shown in the CHO assay [described in Example 8(d)].

These results demonstrate that soluble toxin A repeat immunogen is necessary to induce the production of neutralizing antibodies in chickens, and that the generation of such soluble immunogen is obtained only with a specific expression vector (pMal) containing the toxin A region spanning aa 1870-2680. That is to say, insoluble protein that is subsequently solubilized does not result in a toxin A antigen that will elicit a neutralizing antibody.

### EXAMPLE 18

#### Cloning And Expression Of The *C. difficile* Toxin B Gene

The toxin B gene has been cloned and sequenced: the amino acid sequence deduced from the cloned nucleotide sequence predicts a MW of 269.7 kD for toxin B [Barroso *et al.*, Nucl. Acids Res. 18:4004 (1990)]. The nucleotide sequence of the coding region of the entire toxin B gene is listed in SEQ ID NO:9. The amino acid sequence of the entire toxin B protein is listed in SEQ ID NO:10. The amino acid sequence consisting of amino acid residues 1850 through 2360 of toxin B is listed in SEQ ID NO:11. The amino acid sequence consisting of amino acid residues 1750 through 2360 of toxin B is listed in SEQ ID NO:12.

Given the expense and difficulty of isolating native toxin B protein, it would be advantageous to use simple and inexpensive procaryotic expression systems to produce and purify high levels of recombinant toxin B protein for immunization purposes. Ideally, the isolated recombinant protein would be soluble in order to preserve native antigenicity, since solubilized inclusion body proteins often do not fold into native conformations. Indeed as shown in Example 17, neutralizing antibodies against recombinant toxin A were only obtained when soluble recombinant toxin A polypeptides were used as the immunogen. To allow ease of purification, the recombinant protein should be expressed to levels greater than 1 mg/liter of *E. coli* culture.

To determine whether high levels of recombinant toxin B protein could be produced in *E. coli*, fragments of the toxin B gene were cloned into various prokaryotic expression vectors, and assessed for the ability to express recombinant toxin B protein in *E. coli*. This Example involved (a) cloning of the toxin B gene and (b) expression of the toxin B gene in *E. coli*.



### a) Cloning Of The Toxin B Gene

The toxin B gene was cloned using PCR amplification from *C. difficile* genomic DNA. Initially, the gene was cloned in two overlapping fragments, using primer pairs P5/P6 and P7/P8. The location of these primers along the toxin B gene is shown schematically in Figure 18. The sequence of each of these primers is: P5: 5' TAGAAAAAATGGCAAATGT 3' (SEQ ID NO:11); P6: 5' TTTCATCTTGTA GAGTCAAAG 3' (SEQ ID NO:12); P7: 5' GATGCCACAAGATGATTTAGTG 3' (SEQ ID NO:13); and P8: 5' CTAATTGAGCTGTATCAGGATC 3' (SEQ ID NO:14).

Figure 18 also shows the location of the following primers along the toxin B gene: P9 which consists of the sequence 5' CGGAATTCCTAGAAAAAATGGCAA ATG 3' (SEQ ID NO:15); P10 which consists of the sequence 5' GCTCTAGAATGA CCATAAGCTAGCCA 3' (SEQ ID NO:16); P11 which consists of the sequence 5' CGGAATTCGAGTTGGTAGAAAGGTGGA 3' (SEQ ID NO:17); P13 which consists of the sequence 5' CGGAATTCGGTTATTATCTTAAGGATG 3' (SEQ ID NO:18); and P14 which consists of the sequence 5' CGGAATTCCTTGATAACTGGAT TTGTGAC 3' (SEQ ID NO:19). The amino acid sequence consisting of amino acid residues 1852 through 2362 of toxin B is listed in SEQ ID NO:20. The amino acid sequence consisting of amino acid residues 1755 through 2362 of toxin B is listed in SEQ ID NO:21.

*Clostridium difficile* VPI strain 10463 was obtained from the American Type Culture Collection (ATCC 43255) and grown under anaerobic conditions in brain-heart infusion medium (Becton Dickinson). High molecular-weight *C. difficile* DNA was isolated essentially as described [Wren and Tabaqchali (1987) J. Clin. Microbiol., 25:2402], except 1) 100 µg/ml proteinase K in 0.5% SDS was used to disrupt the bacteria and 2) cetyltrimethylammonium bromide (CTAB) precipitation [as described by Ausubel *et al.*, Eds., *Current Protocols in Molecular Biology*, Vol. 2 (1989) Current Protocols] was used to remove carbohydrates from the cleared lysate. Briefly, after disruption of the bacteria with proteinase K and SDS, the solution is adjusted to approximately 0.7 M NaCl by the addition of a 1/7 volume of 5M NaCl. A 1/10 volume of CTAB/NaCl (10% CTAB in 0.7 M NaCl) solution was added and the solution was mixed thoroughly and incubated 10 min at 65°C. An equal volume of chloroform/isoamyl alcohol (24:1) was added and the phases were thoroughly mixed. The organic and aqueous phases were separated by centrifugation in a microfuge for 5 min. The aqueous supernatant was removed and extracted with phenol/chloroform/ isoamyl alcohol (25:24:1). The phases were separated by centrifugation in a microfuge for 5 min. The

supernatant was transferred to a fresh tube and the DNA was precipitated with isopropanol. The DNA precipitate was pelleted by brief centrifugation in a microfuge. The DNA pellet was washed with 70% ethanol to remove residual CTAB. The DNA pellet was then dried and redissolved in TE buffer (10 mM Tris-HCl pH8.0, 1 mM EDTA). The integrity and  
5 yield of genomic DNA was assessed by comparison with a serial dilution of uncut lambda DNA after electrophoresis on an agarose gel.

Toxin B fragments were cloned by PCR utilizing a proofreading thermostable DNA polymerase [native *Pfu* polymerase (Stratagene)]. The high fidelity of this polymerase reduces the mutation problems associated with amplification by error prone polymerases (*e.g.*,  
10 *Taq* polymerase). PCR amplification was performed using the PCR primer pairs P5 (SEQ ID NO:11) with P6 (SEQ ID NO:12) and P7 (SEQ ID NO:13) with P8 (SEQ ID NO:14) in 50 µl reactions containing 10 mM Tris-HCl pH8.3, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 200 µM of each dNTP, 0.2 µM each primer, and 50 ng *C. difficile* genomic DNA. Reactions were overlaid with 100 µl mineral oil, heated to 94°C for 4 min, 0.5µl native *Pfu* polymerase  
15 (Stratagene) was added, and the reactions were cycled 30 times at 94°C for 1 min, 50°C for 1 min, 72°C (2 min for each kb of sequence to be amplified), followed by 10 min at 72°C. Duplicate reactions were pooled, chloroform extracted, and ethanol precipitated. After washing in 70% ethanol, the pellets were resuspended in 50 µl TE buffer (10 mM Tris-HCl pH8.0, 1 mM EDTA).

The P5/P6 amplification product was cloned into pUC19 as outlined below. 10µl  
20 aliquots of DNA were gel purified using the Prep-a-Gene kit (BioRad), and ligated to *Sma*I restricted pUC19 vector. Recombinant clones were isolated and confirmed by restriction digestion using standard recombinant molecular biology techniques (Sambrook *et al.*, 1989). Inserts from two independent isolates were identified in which the toxin B insert was oriented  
25 such that the vector *Bam*HI and *Sac*I sites were 5' and 3' oriented, respectively (pUCB10-1530). The insert-containing *Bam*HI/*Sac*I fragment was cloned into similarly cut pET23a-c vector DNA, and protein expression was induced in small scale cultures (5 ml) of identified clones.

Total protein extracts were isolated, resolved on SDS-PAGE gels, and toxin B protein  
30 identified by Western analysis utilizing a goat anti-toxin B affinity purified antibody (Tech Lab). Procedures for protein induction, SDS-PAGE, and Western blot analysis were performed as described in Williams *et al.* (1995), *supra*. In brief, 5 ml cultures of bacteria grown in 2XYT containing 100 µg/ml ampicillin containing the appropriate recombinant clone

were induced to express recombinant protein by addition of IPTG to 1mM. The *E. coli* hosts used were: BL21(DE3) or BL21(DE3)LysS (Novagen) for pET plasmids.

Cultures were induced by the addition of IPTG to a final concentration of 1.0 mM when the cell density reached 0.5 OD<sub>600</sub>, and induced protein was allowed to accumulate for two hrs after induction. Protein samples were prepared by pelleting 1 ml aliquots of bacteria by centrifugation (1 min in microfuge), and resuspension of the pelleted bacteria in 150 µl of 2X SDS-PAGE sample buffer (0.125 mM Tris-HCl pH 6.8, 2 mM EDTA, 6% SDS, 20% glycerol, 0.025% bromophenol blue; β-mercaptoethanol is added to 5% before use). The samples were heated to 95°C for 5 min, then cooled and 5 or 10 µls loaded on 7.5% SDS-PAGE gels. High molecular weight protein markers (BioRad) were also loaded, to allow estimation of the MW of identified fusion proteins. After electrophoresis, protein was detected either generally by staining the gels with Coomassie Blue, or specifically, by blotting to nitrocellulose for Western blot detection of specific immunoreactive protein. The MW of induced toxin B reactive protein allowed the integrity of the toxin B reading frame to be determined.

The pET23b recombinant (pPB10-1530) expressed high MW recombinant toxin B reactive protein, consistent with predicted values. This confirmed that frame terminating errors had not occurred during PCR amplification. A pET23b expression clone containing the 10-1750aa interval of the toxin B gene was constructed, by fusion of the *EcoRV-SpeI* fragment of the P7/P8 amplification product to the P5-*EcoRV* interval of the P5/P6 amplification product (see Figure 18) in pPB10-1530. The integrity of this clone (pPB10-1750) was confirmed by restriction mapping, and Western blot detection of expressed recombinant toxin B protein. Levels of induced protein from both pPB10-1530 and pPB10-1750 were too low to facilitate purification of usable amounts of recombinant toxin B protein. The remaining 1750-2360 aa interval was directly cloned into pMAL or pET expression vectors from the P7/P8 amplification product as described below.

## **b) Expression Of The Toxin B Gene**

### **i) Overview Of Expression Methodologies**

Fragments of the toxin B gene were expressed as either native or fusion proteins in *E. coli*. Native proteins were expressed in either the pET23a-c or pET16b expression vectors (Novagen). The pET23 vectors contain an extensive polylinker sequence in all three reading frames (a-c vectors) followed by a C-terminal poly-histidine repeat. The pET16b vector

contains a N-terminal poly-histidine sequence immediately 5' to a small polylinker. The poly-histidine sequence binds to Ni-Chelate columns and allows affinity purification of tagged target proteins [Williams *et al.* (1995), *supra*]. These affinity tags are small (10 aa for pET16b, 6 aa for pET23) allowing the expression and affinity purification of native proteins with only limited amounts of foreign sequences.

An N-terminal histidine-tagged derivative of pET16b containing an extensive cloning cassette was constructed to facilitate cloning of N-terminal poly-histidine tagged toxin B expressing constructs. This was accomplished by replacement of the promoter region of the pET23a and b vectors with that of the pET16b expression vector. Each vector was restricted with *Bgl*II and *Nde*I, and the reactions resolved on a 1.2 % agarose gel. The pET16b promoter region (contained in a 200 bp *Bgl*II-*Nde*I fragment) and the promoter-less pET23 a or b vectors were cut from the gel, mixed and Prep-A-Gene (BioRad) purified. The eluted DNA was ligated, and transformants screened for promoter replacement by *Nco*I digestion of purified plasmid DNA (the pET16b promoter contains this site, the pET23 promoter does not). These clones (denoted pETHisa or b) contain the pET16b promoter (consisting of a pT7-lac promoter, ribosome binding site and poly-histidine (10aa) sequence) fused at the *Nde*I site to the extensive pET23 polylinker.

All MBP fusion proteins were constructed and expressed in the pMAL<sup>TM</sup>-c or pMAL<sup>TM</sup>-p2 vectors (New England Biolabs) in which the protein of interest is expressed as a C-terminal fusion with MBP. All pET plasmids were expressed in either the BL21(DE3) or BL21(DE3)lysS expression hosts, while pMal plasmids were expressed in the BL21 host.

Large scale (500 mls to 1 liter) cultures of each recombinant were grown in 2X YT broth, induced, and soluble protein fractions were isolated as described [Williams, *et al.* (1995), *supra*]. The soluble protein extracts were affinity chromatographed to isolate recombinant fusion protein, as described [Williams *et al.*, (1995) *supra*]. In brief, extracts containing tagged pET fusions were chromatographed on a nickel chelate column, and eluted using imidazole salts or low pH (pH 4.0) as described by the distributor (Novagen or Qiagen). Extracts containing soluble pMAL fusion protein were prepared and chromatographed in PBS buffer over an amylose resin (New England Biolabs) column, and eluted with PBS containing 10 mM maltose as described [Williams *et al.* (1995), *supra*].

## ii) Overview Of Toxin B Expression

In both large expression constructs described in (a) above, only low level (*i.e.*, less than 1 mg/liter of intact or nondegraded recombinant protein) expression of recombinant protein was detected. A number of expression constructs containing smaller fragments of the toxin B gene were then constructed, to determine if small regions of the gene can be expressed to high levels (*i.e.*, greater than 1 mg/liter intact protein) without extensive protein degradation. All were constructed by in frame fusions of convenient toxin B restriction fragments to either the pMAL-c, pET23a-c, pET16b or pET11sa-b expression vectors, or by engineering restriction sites at specific locations using PCR amplification [using the same conditions described in (a) above]. In all cases, clones were verified by restriction mapping, and, where indicated, DNA sequencing.

Protein preparations from induced cultures of each of these constructs were analyzed, by SDS-PAGE, to estimate protein stability (Coomassie Blue staining) and immunoreactivity against anti-toxin B specific antiserum (Western analysis). Higher levels of intact (*i.e.*, nondegraded), full length fusion proteins were observed with the smaller constructs as compared with the larger recombinants, and a series of expression constructs spanning the entire toxin B gene were constructed (Figures 18, 19 and 20 and Table 23).

Constructs that expressed significant levels of recombinant toxin B protein (greater than 1 mg/liter intact recombinant protein) in *E. coli* were identified and a series of these clones that spans the toxin B gene are shown in Figure 19 and summarized in Table 23. These clones were utilized to isolate pure toxin B recombinant protein from the entire toxin B gene. Significant protein yields were obtained from pMAL expression constructs spanning the entire toxin B gene, and yields of full length soluble fusion protein ranged from an estimated 1 mg/liter culture (pMB1100-1530) to greater than 20 mg/liter culture (pMB1750-2360).

Representative purifications of MBP and poly-histidine-tagged toxin B recombinants are shown in Figures 21 and 22. Figure 21 shows a Coomassie Blue stained 7.5% SDS-PAGE gel on which various protein samples extracted from bacteria harboring pMB1850-2360 were electrophoresed. Samples were loaded as follows: Lane 1: protein extracted from uninduced culture; Lane 2: induced culture protein; Lane 3: total protein from induced culture after sonication; Lane 4: soluble protein; and Lane 5: eluted affinity purified protein. Figure 22 depicts the purification of recombinant proteins expressed in bacteria harboring either pPB1850-2360 (Lanes 1-3) or pPB1750-2360 (Lanes 4-6). Samples were loaded as follows: uninduced total protein (Lanes 1 and 4); induced total protein (Lanes 2 and 5); and eluted

affinity purified protein (Lanes 3 and 6). The broad range molecular weight protein markers (BioRad) are shown in Lane 7.

Thus, although high level expression was not attained using large expression constructs from the toxin B gene, usable levels of recombinant protein were obtained by isolating induced protein from a series of smaller pMAL expression constructs that span the entire toxin B gene.

These results represent the first demonstration of the feasibility of expressing recombinant toxin B protein to high levels in *E. coli*. As well, expression of small regions of the putative ligand binding domain (repeat region) of toxin B as native protein yielded insoluble protein, while large constructs, or fusions to MBP were soluble (Figure 19), demonstrating that specific methodologies are necessary to produce soluble fusion protein from this interval.

### iii) Clone Construction And Expression Details

A portion of the toxin B gene containing the toxin B repeat region [amino acid residues 1852-2362 of toxin B (SEQ ID NO:20)] was isolated by PCR amplification of this interval of the toxin B gene from *C. difficile* genomic DNA. The sequence, and location within the toxin B gene, of the two PCR primers [P7 (SEQ ID NO:13) and P8 (SEQ ID NO:14)] used to amplify this region are shown in Figure 18.

DNA from the PCR amplification was purified by chloroform extraction and ethanol precipitation as described above. The DNA was restricted with *SpeI*, and the cleaved DNA was resolved by agarose gel electrophoresis. The restriction digestion with *SpeI* cleaved the 3.6 kb amplification product into a 1.8 kb doublet band. This doublet band was cut from the gel and mixed with appropriately cut, gel purified pMALc or pET23b vector. These vectors were prepared by digestion with *HindIII*, filling in the overhanging ends using the Klenow enzyme, and cleaving with *XbaI* (pMALc) or *NheI* (pET23b). The gel purified DNA fragments were purified using the Prep-A-Gene kit (BioRad) and the DNA was ligated, transformed and putative recombinant clones analyzed by restriction mapping.

pET and pMal clones containing the toxin B repeat insert (aa interval 1750-2360 of toxin B) were verified by restriction mapping, using enzymes that cleaved specific sites within the toxin B region. In both cases fusion of the toxin B *SpeI* site with either the compatible *XbaI* site (pMal) or compatible *NheI* site (pET) is predicted to create an in frame fusion. This was confirmed in the case of the pMB1750-2360 clone by DNA sequencing of the clone

junction and 5' end of the toxin B insert using a MBP specific primer (New England Biolabs). In the case of the pET construct, the fusion of the blunt ended toxin B 3' end to the filled *HindIII* site should create an in-frame fusion with the C-terminal poly-histidine sequence in this vector. The pPB1750-2360 clone selected had lost, as predicted, the *HindIII* site at this clone junction; this eliminated the possibility that an additional adenosine residue was added to the 3' end of the PCR product by a terminal transferase activity of the *Pfu* polymerase, since fusion of this adenosine residue to the filled *HindIII* site would regenerate the restriction site (and was observed in several clones).

One liter cultures of each expression construct were grown, and fusion protein purified by affinity chromatography on either an amylose resin column (pMAL constructs; resin supplied by New England Biolabs) or Ni-chelate column (pET constructs; resin supplied by Qiagen or Novagen) as described [Williams *et al.* (1995), *supra*]. The integrity and purity of the fusion proteins were determined by Coomassie staining of SDS-PAGE protein gels as well as Western blot analysis with either an affinity purified goat polyclonal antiserum (Tech Lab), or a chicken polyclonal PEG prep. raised against the toxin B protein (CTB) as described above in Example 8. In both cases, affinity purification resulted in yields in excess of 20 mg protein per liter culture, of which greater than 90% was estimated to be full-length recombinant protein. It should be noted that the poly-histidine affinity tagged protein was released from the Qiagen Ni-NTA resin at low imidazole concentration (60 mM), necessitating the use of a 40 mM imidazole rather than a 60 mM imidazole wash step during purification.

A periplasmically secreted version of pMB1750-2360 was constructed by replacement of the promoter and MBP coding region of this construct with that from a related vector (pMAL<sup>TM</sup>-p2; New England Biolabs) in which a signal sequence is present at the N-terminus of the MBP, such that fusion protein is exported. This was accomplished by substituting a *BglII-EcoRV* promoter fragment from pMAL-p2 into pMB1750-2360. The yields of secreted, affinity purified protein (recovered from osmotic shock extracts as described by Riggs in *Current Protocols in Molecular Biology*, Vol. 2, Ausubel, *et al.*, Eds. (1989), Current Protocols, pp. 16.6.1 - 16.6.14] from this vector (pMBp1750-2360) were 6.5 mg/liter culture, of which 50% was estimated to be full-length fusion protein.

The interval was also expressed in two non-overlapping fragments. pMB1750-1970 was constructed by introduction of a frameshift into pMB1750-2360, by restriction with *HindIII*, filling in the overhanging ends and religation of the plasmid. Recombinant clones

were selected by loss of the *Hind*III site, and further restriction map analysis. Recombinant protein expression from this vector was more than 20 mg/liter of greater than 90% pure protein.

5 The complementary region was expressed in pMB1970-2360. This construct was created by removal of the 1750-1970 interval of pMB1750-2360. This was accomplished by restriction of this plasmid with *Eco*RI (in the pMalc polylinker 5' to the insert) and III, filling in the overhanging ends, and religation of the plasmid. The resultant plasmid, pMB1970-2360, was made using both intracellularly and secreted versions of the pMB1750-2360 vector.

10 No fusion protein was secreted in the pMBp1970-2360 version, perhaps due to a conformational constraint that prevents export of the fusion protein. However, the intracellularly expressed vector produced greater than 40mg/liter of greater than 90% full-length fusion protein.

15 Constructs to precisely express the toxin B repeats in either pMalc (pMB1850-2360) or pET16b (pPB1850-2360) were constructed as follows. The DNA interval including the toxin B repeats was PCR amplified as described above utilizing PCR primers P14 (SEQ ID NO:19) and P8 (SEQ ID NO:14). Primer P14 adds a *Eco*RI site immediately flanking the start of the toxin B repeats.

20 The amplified fragment was cloned into the pT7 Blue T-vector (Novagen) and recombinant clones in which single copies of the PCR fragment were inserted in either orientation were selected (pT71850-2360) and confirmed by restriction mapping. The insert was excised from two appropriately oriented independently isolated pT71850-2360 plasmids, with *Eco*RI (5' end of repeats) and *Pst*I (in the flanking polylinker of the vector), and cloned into *Eco*RI/*Pst*I cleaved pMalc vector. The resulting construct (pMB1850-2360) was confirmed by restriction analysis, and yielded 20 mg/l of soluble fusion protein [greater than 25 90% intact (*i.e.*, nondegraded)] after affinity chromatography. Restriction of this plasmid with *Hind*III and religation of the vector resulted in the removal of the 1970-2360 interval. The resultant construct (pMB1850-1970) expressed greater than 70 mg/liter of 90% full length fusion protein.



The pPB1850-2360 construct was made by cloning a *EcoRI* (filled with Klenow)-*BamHI* fragment from a pT71850-2360 vector (opposite orientation to that used in the pMB1850-2360 construction) into *NdeI* (filled)/*BamHI* cleaved pET16b vector. Yields of affinity purified soluble fusion protein were 15 mg/liter, of greater than 90% full length fusion protein.

Several smaller expression constructs from the 1750-2070 interval were also constructed in His-tagged pET vectors, but expression of these plasmids in the BL21 (DE3) host resulted in the production of high levels of mostly insoluble protein (see Table 23 and Figure 19). These constructs were made as follows.

pPB1850-1970 was constructed by cloning a *BglII-HindIII* fragment of pPB1850-2360 into *BglII/HindIII* cleaved pET23b vector. pPB1850-2070 was constructed by cloning a *BglII-PvuII* fragment of pPB1850-2360 into *BglII/HincII* cleaved pET23b vector. pPB1750-1970(c) was constructed by removal of the internal *HindIII* fragment of a pPB1750-2360 vector in which the vector *HindIII* site was regenerated during cloning (presumably by the addition of an A residue to the amplified PCR product by terminal transferase activity of *Pfu* polymerase). The pPB1750-1970(n) construct was made by insertion of the insert containing the *NdeI-HindIII* fragment of pPB1750-2360 into identically cleaved pETHisb vector. All constructs were confirmed by restriction digestion.

An expression construct that directs expression of the 10-470 aa interval of toxin B was constructed in the pMalc vector as follows. A *NheI* (a site 5' to the insert in the pET23 vector)-*AflIII* (filled) fragment of the toxin B gene from pPB10-1530 was cloned into *NheI* (compatible with *NheI*)/*HindIII* (filled) pMalc vector. The integrity of the construct (pMB10-470) was verified by restriction mapping and DNA sequencing of the 5' clone junction using a MBP specific DNA primer (New England Biolabs). However, all expressed protein was degraded to the MBP monomer MW.

A second construct spanning this interval (aa 10-470) was constructed by cloning the PCR amplification product from a reaction containing the P9 (SEQ ID NO:15) and P10 (SEQ ID NO:16) primers (Figure 18) into the pETHisA vector. This was accomplished by cloning the PCR product as an *EcoRI*-blunt fragment into *EcoRI-HincII* restricted vector DNA; recombinant clones were verified by restriction mapping. Although this construct (pPB10-520) allowed expression and purification (utilizing the N-terminal polyhistidine affinity tag) of intact fusion protein, yields were estimated at less than 500 µg per liter culture.

Higher yield of recombinant protein from this interval (aa 10-520) were obtained by expression of the interval in two overlapping clones. The 10-330aa interval was cloned in both pETHisa and pMalc vectors, using the *Bam*HI-*Afl*III (filled) DNA fragment from pPB10-520. This fragment was cloned into *Bam*HI-*Hind*III (filled) restricted pMalc or *Bam*HI-*Hinc*II restricted pETHisa vector. Recombinant clones were verified by restriction mapping. High level expression of either insoluble (pET) or soluble (pMal) fusion protein was obtained. Total yields of fusion protein from the pMB10-330 construct (Figure 18) were 20 mg/liter culture, of which 10% was estimated to be full-length fusion protein. Although yields of this interval were higher and >90% full-length recombinant protein produced when expressed from the pET construct, the pMal fusion was utilized since the expressed protein was soluble and thus more likely to have the native conformation.

The pMB260-520 clone was constructed by cloning *Eco*RI-*Xba*I cleaved amplified DNA from a PCR reaction containing the P11 (SEQ ID NO:17) and P10 (SEQ ID NO:16) DNA primers (Figure 18) into similarly restricted pMalc vector. Yields of affinity purified protein were 10 mg/liter, of which approximately 50% was estimated to be full-length recombinant protein.

The aa510-1110 interval was expressed as described below. This entire interval was expressed as a pMal fusion by cloning the *Nhe*I-*Hind*III fragment of pUCB10-1530 into *Xba*I-*Hind*III cleaved pMalc vector. The integrity of the construct (pMB510-1110) was verified by restriction mapping and DNA sequencing of the 5' clone junction using a MBP specific DNA primer. The yield of affinity purified protein was 25 mg/liter culture, of which 5% was estimated to be full-length fusion protein (1 mg/liter).

To attempt to obtain higher yields, this region was expressed in two fragments (aa510-820, and 820-1110) in the pMalc vector. The pMB510-820 clone was constructed by insertion of a *Sac*I (in the pMalc polylinker 5' to the insert)-*Hpa*I DNA fragment from pMB510-1110 into *Sac*I/*Stu*I restricted pMalc vector. The pMB820-1110 vector was constructed by insertion of the *Hpa*I-*Hind*III fragment of pUCB10-1530 into *Bam*HI (filled)-*Hind*III cleaved pMalc vector. The integrity of these constructs were verified by restriction mapping and DNA sequencing of the 5' clone junction using a MBP specific DNA primer.

Recombinant protein expressed from the pMB510-820 vector was highly unstable. However, high levels (20 mg/liter) of >90% full-length fusion protein were obtained from the pMB820-1105 construct. The combination of partially degraded pMB510-1110 protein

(enriched for the 510-820 interval) with the pMB820-1110 protein provides usable amounts of recombinant antigen from this interval.

The aa1100-1750 interval was expressed as described below. The entire interval was expressed in the pMalc vector from a construct in which the *AccI*(filled)-*SpeI* fragment of pPB10-1750 was inserted into *StuI/XbaI* (*XbaI* is compatible with *SpeI*; *StuI* and filled *AccI* sites are both blunt ended) restricted pMalc. The integrity of this construct (pMB1100-1750) was verified by restriction mapping and DNA sequencing of the clone junction using a MBP specific DNA primer. Although 15 mg/liter of affinity purified protein was isolated from cells harboring this construct, the protein was greater than 99% degraded to MBP monomer size.

A smaller derivative of pMB1100-1750 was constructed by restriction of pMB1100-1750 with *AflII* and *Sall* (in the pMalc polylinker 3' to the insert), filling in the overhanging ends, and religating the plasmid. The resultant clone (verified by restriction digestion and DNA sequencing) has deleted the aa1530-1750 interval, was designated pMB1100-1530. pMB1100-1530 expressed recombinant protein at a yield of greater than 40 mg/liter, of which 5% was estimated to be full-length fusion protein.

Three constructs were made to express the remaining interval. Initially, a *BspHI* (filled)-*SpeI* fragment from pPB10-1750 was cloned into *EcoRI*(filled)/*XbaI* cleaved pMalc vector. The integrity of this construct (pMB1570-1750) was verified by restriction mapping and DNA sequencing of the 5' clone junction using a MBP specific DNA primer. Expression of recombinant protein from this plasmid was very low, approximately 3 mg affinity purified protein per liter, and most was degraded to MBP monomer size. This region was subsequently expressed from a PCR amplified DNA fragment. A PCR reaction utilizing primers P13 [SEQ ID NO:18; P13 was engineered to introduce an *EcoRI* site 5' to amplified toxin B sequences] and P8 (SEQ ID NO:14) was performed on *C. difficile* genomic DNA as described above. The amplified fragment was cleaved with *EcoRI* and *SpeI*, and cloned into *EcoRI/XbaI* cleaved pMalc vector. The resultant clone (pMB1530-1750) was verified by restriction map analysis, and recombinant protein was expressed and purified. The yield was greater than 20 mg protein per liter culture and it was estimated that 25% was full-length fusion protein; this was a significantly higher yield than the original pMB1570-1750 clone. The insert of pMB1530-1750 (in a *EcoRI-Sall* fragment) was transferred to the pETHisa vector (*EcoRI/XhoI* cleaved, *XhoI* and *Sall* ends are compatible). No detectable fusion protein was purified on Ni-Chelate columns from soluble lysates of cells induced to express fusion protein from this construct.

TABLE 23

Summary Of Toxin B Expression Constructs<sup>a</sup>

Clone	Affinity Tag	Yield (mg/liter)	% Full Length
pPB10-1750	none	low (estimated from Western blot hyb.)	?
pPB10-1530	none	low (as above)	?
pMB10-470	MBP	15mg/l	0%
pPB10-520	poly-his	0.5mg/l	20%
pPB10-330	poly-his	20mg/l (insoluble)	90%
<i>pMB10-330</i>	<i>MBP</i>	<i>20mg/l</i>	<i>100%</i>
<i>pMB260-520</i>	<i>MBP</i>	<i>10mg/l</i>	<i>50%</i>
<i>pMB510-1110</i>	<i>MBP</i>	<i>25mg/l</i>	<i>5%</i>
pMB510-820	MBP	degraded (by Western blot hyb)	
<i>pMB820-1110</i>	<i>MBP</i>	<i>20mg/l</i>	<i>90%</i>
pMB1100-1750	MBP	15mg/l	0%
<i>pMB1100-1530</i>	<i>MBP</i>	<i>40mg/l</i>	<i>5%</i>
pMB1570-1750	MBP	3mg/l	5%
pPB1530-1750	poly-his	no purified protein detected	?
<i>pMB1530-1750</i>	<i>MBP</i>	<i>20mg/l</i>	<i>25%</i>
<i>pMB1750-2360</i>	<i>MBP</i>	<i>20mg/l</i>	<i>90%</i>
pMBp1750-2360	MBP	6.5mg/l (secreted)	50%
pPB1750-2360	poly-his	20mg/l	90%
pMB1750-1970	MBP	20mg/l	90%
pMB1970-2360	MBP	40mg/l	90%
pMBp1970-2360	MBP	(no secretion)	NA
pMB1850-2360	MBP	20mg/l	90%
pPB1850-2360	poly-his	15mg/l	90%
pMB1850-1970	MBP	70mg/l	90%
pPB1850-1970	poly-his	10mg/l (insoluble)	90%
pPB1850-2070	poly-his	10mg/l (insoluble)	90%
pPB1750-1970(c)	poly-his	10mg/l (insoluble)	90%
pPB1750-1970(n)	poly-his	10mg/l (insoluble)	90%

<sup>a</sup> Clones in italics are clones currently utilized to purify recombinant protein from each selected interval.

occurs with the CTB antibody-recombinant mixture, that recombinant contains only weak or non-neutralizing epitopes of toxin B. This assay was performed as follows.

Antibodies against CTB were generated in egg laying Leghorn hens as described in Example 8. The lethal dosage (LD<sub>100</sub>) of *C. difficile* toxin B when delivered I.P. into 40g female Golden Syrian hamsters (Charles River) was determined to be 2.5 to 5 µg. Antibodies generated against CTB and purified by PEG precipitation could completely protect the hamsters at the I.P. dosage determined above. The minimal amount of CTB antibody needed to afford good protection against 5 µg of CTB when injected I.P. into hamsters was also determined (1X PEG prep). These experiments defined the parameters needed to test whether a given recombinant protein could deplete protective CTB antibodies.

The cloned regions tested for neutralizing ability cover the entire toxin B gene and were designated as Intervals (INT) 1 through 5 (see Figure 19). Approximately equivalent final concentrations of each recombinant polypeptide were tested. The following recombinant polypeptides were used: 1) a mixture of intervals 1 and 2 (INT-1, 2); 2) a mixture of Intervals 4 and 5 (INT-4, 5) and 3) Interval 3 (INT-3). Recombinant proteins (each at about 1 mg total protein) were first preincubated with a final CTB antibody concentration of 1X [*i.e.*, pellet dissolved in original yolk volume as described in Example 1(c)] in a final volume of 5 mls for 1 hour at 37°C. Twenty-five µg of CTB (at a concentration of 5 µg/ml), enough CTB to kill 5 hamsters, was then added and the mixture was then incubated for 1 hour at 37°C. Five, 40g female hamsters (Charles River) in each treatment group were then each given 1 ml of the mixture I.P. using a tuberculin syringe with a 27 gauge needle. The results of this experiment are shown in Table 24.

TABLE 24

Binding Of Neutralizing Antibodies By INT 3 Protein

Treatment Group <sup>1</sup>	Number Of Animals Alive	Number Of Animals Dead
CTB antibodies	3	2
CTB antibodies + INT1,2	3	2
CTB antibodies + INT4,5	3	2
CTB antibodies + INT 3	0	5

<sup>1</sup> *C. difficile* toxin B (CTB) was added to each group.

As shown in Table 24, the addition of recombinant proteins from INT-1, 2 or INT-4, 5 had no effect on the *in vivo* protective ability of the CTB antibody preparation compared to

**EXAMPLE 19****Identification, Purification And Induction Of Neutralizing  
Antibodies Against Recombinant *C. difficile* Toxin B Protein**

5 To determine whether recombinant toxin B polypeptide fragments can generate neutralizing antibodies, typically animals would first be immunized with recombinant proteins and anti-recombinant antibodies are generated. These anti-recombinant protein antibodies are then tested for neutralizing ability *in vivo* or *in vitro*. Depending on the immunogenic nature of the recombinant polypeptide, the generation of high-titer antibodies against that protein 10 may take several months. To accelerate this process and identify which recombinant polypeptide(s) may be the best candidate to generate neutralizing antibodies, depletion studies were performed. Specifically, recombinant toxin B polypeptide were pre-screened by testing whether they have the ability to bind to protective antibodies from a CTB antibody preparation and hence deplete those antibodies of their neutralizing capacity. Those 15 recombinant polypeptides found to bind CTB, were then utilized to generate neutralizing antibodies. This Example involved: a) identification of recombinant sub-regions within toxin B to which neutralizing antibodies bind; b) identification of toxin B sub-region specific antibodies that neutralize toxin B *in vivo*; and c) generation and evaluation of antibodies reactive to recombinant toxin B polypeptides.

20

**a) Identification Of Recombinant Sub-Regions Within Toxin B  
To Which Neutralizing Antibodies Bind**

Sub-regions within toxin B to which neutralizing antibodies bind were identified by utilizing recombinant toxin B proteins to deplete protective antibodies from a polyclonal pool 25 of antibodies against native *C. difficile* toxin B. An *in vivo* assay was developed to evaluate protein preparations for the ability to bind neutralizing antibodies. Recombinant proteins were first pre-mixed with antibodies directed against native toxin B (CTB antibody; see Example 8) and allowed to react for one hour at 37°C. Subsequently, *C. difficile* toxin B (CTB; Tech Lab) was added at a concentration lethal to hamsters and incubated for another hour at 37°C. 30 After incubation this mixture was injected intraperitoneally (IP) into hamsters. If the recombinant polypeptide contains neutralizing epitopes, the CTB antibodies will lose its ability to protect the hamsters against death from CTB. If partial or complete protection

the CTB antibody preparation alone. In contrast, INT-3 recombinant polypeptide was able to remove all of the toxin B neutralizing ability of the CTB antibodies as demonstrated by the death of all the hamsters in that group.

The above experiment was repeated, using two smaller expressed fragments (pMB 1750-1970 and pMB 1970-2360, see Figure 19) comprising the INT-3 domain to determine if that domain could be further subdivided into smaller neutralizing epitopes. In addition, full-length INT-3 polypeptide expressed as a nickel tagged protein (pPB1750-2360) was tested for neutralizing ability and compared to the original INT-3 expressed MBP fusion (pMB1750-2360). The results are shown in Table 25.

TABLE 25

Removal Of Neutralizing Antibodies By Repeat Containing Proteins

Treatment Group <sup>1</sup>	Number Of Animals Alive	Number Of Animals Dead
CTB antibodies	5	0
CTB antibodies + pPB1750-2360	0	5
CTB antibodies + pMB1750-2360	0	5
CTB antibodies + pMB1970-2360	3	2
CTB antibodies + pMB1750-1970	2	3

<sup>1</sup> *C. difficile* toxin B (CTB) was added to each group.

The results summarized in Table 25 indicate that the smaller polypeptide fragments within the INT-3 domain, pMB1750-1970 and pMB1970-2360, partially lose the ability to bind to and remove neutralizing antibodies from the CTB antibody pool. These results demonstrate that the full length INT-3 polypeptide is required to completely deplete the CTB antibody pool of neutralizing antibodies. This experiment also shows that the neutralization epitope of INT-3 can be expressed in alternative vector systems and the results are independent of the vector utilized or the accompanying fusion partner.

Other Interval 3 specific proteins were subsequently tested for the ability to remove neutralizing antibodies within the CTB antibody pool as described above. The Interval 3 specific proteins used in these studies are summarized in Figure 23. In Figure 23 the following abbreviations are used: pP refers to the pET23 vector; pM refers to the pMALc vector; B refers to toxin B; the numbers refer to the amino acid interval expressed in the clone. The solid black ovals represent the MBP; and HHH represents the poly-histidine tag.

Only recombinant proteins comprising the entire toxin B repeat domain (pMB1750-2360, pPB1750-2360 and pPB1850-2360) can bind and completely remove neutralizing antibodies from the CTB antibody pool. Recombinant proteins comprising only a portion of the toxin B repeat domain were not capable of completely removing neutralizing antibodies from the CTB antibody pool (pMB1750-1970 and pMB1970-2360 could partially remove neutralizing antibodies while pMB1850-1970 and pPB1850-2070 failed to remove any neutralizing antibodies from the CTB antibody pool).

The above results demonstrate that only the complete ligand binding domain (repeat region) of the toxin B gene can bind and completely remove neutralizing antibodies from the CTB antibody pool. These results demonstrate that antibodies directed against the entire toxin B repeat region are necessary for *in vivo* toxin neutralization (see Figure 23: only the recombinant proteins expressed by the pMB1750-2360, pPB1750-2360 and pPB1850-2360 vectors are capable of completely removing the neutralizing antibodies from the CTB antibody pool).

These results represent the first indication that the entire repeat region of toxin B would be necessary for the generation of antibodies capable of neutralizing toxin B, and that sub-regions may not be sufficient to generate maximal titers of neutralizing antibodies.

#### **b) Identification Of Toxin B Sub-Region Specific Antibodies That Neutralize Toxin B *In Vivo***

To determine if antibodies directed against the toxin B repeat region are sufficient for neutralization, region specific antibodies within the CTB antibody preparation were affinity purified, and tested for *in vivo* neutralization. Affinity columns containing recombinant toxin B repeat proteins were made as described below. A separate affinity column was prepared using each of the following recombinant toxin B repeat proteins: pPB1750-2360, pPB1850-2360, pMB1750-1970 and pMB1970-2360.

For each affinity column to be made, four ml of PBS-washed Actigel resin (Sterogene) was coupled overnight at room temperature with 5-10 mg of affinity purified recombinant protein (first extensively dialyzed into PBS) in 15 ml tubes (Falcon) containing a 1/10 final volume Ald-coupling solution (1 M sodium cyanoborohydride). Aliquots of the supernatants from the coupling reactions, before and after coupling, were assessed by Coomassie staining of 7.5% SDS-PAGE gels. Based on protein band intensities, in all cases greater than 30% coupling efficiencies were estimated. The resins were poured into 10 ml columns (BioRad).



washed extensively with PBS, pre-eluted with 4M guanidine-HCl (in 10 mM Tris-HCl, pH 8.0) and reequilibrated in PBS. The columns were stored at 4°C.

5 Aliquots of a CTB IgY polyclonal antibody preparation (PEG prep) were affinity purified on each of the four columns as described below. The columns were hooked to a UV monitor (ISCO), washed with PBS and 40 ml aliquots of a 2X PEG prep (filter sterilized using a 0.45  $\mu$  filter) were applied. The columns were washed with PBS until the baseline value was re-established. The columns were then washed with BBSween to elute nonspecifically binding antibodies, and reequilibrated with PBS. Bound antibody was eluted from the column in 4M guanidine-HCl (in 10mM Tris-HCl, pH8.0). The eluted antibody was immediately dialyzed against a 100-fold excess of PBS at 4°C for 2 hrs. The samples were then dialyzed extensively against at least 2 changes of PBS, and affinity purified antibody was collected and stored at 4°C. The antibody preparations were quantified by UV absorbance. The elution volumes were in the range of 4-8 ml. All affinity purified stocks contained similar total antibody concentrations, ranging from 0.25-0.35% of the total protein applied to the columns.

15 The ability of the affinity purified antibody preparations to neutralize toxin B *in vivo* was determined using the assay outlined in a) above. Affinity purified antibody was diluted 1:1 in PBS before testing. The results are shown in Table 26.

20 In all cases similar levels of toxin neutralization was observed, such that lethality was delayed in all groups relative to preimmune controls. This result demonstrates that antibodies reactive to the repeat region of the toxin B gene are sufficient to neutralize toxin B *in vivo*. The hamsters will eventually die in all groups, but this death is maximally delayed with the CTB PEG prep antibodies. Thus neutralization with the affinity purified (AP) antibodies is not as complete as that observed with the CTB prep before affinity chromatography. This result may be due to loss of activity during guanidine denaturation (during the elution of the antibodies from the affinity column) or the presence of antibodies specific to other regions of the toxin B gene that can contribute to toxin neutralization (present in the CTB PEG prep).

TABLE 26

Neutralization Of Toxin B By Affinity Purified Antibodies

Treatment group <sup>a</sup>	Number Animals Alive <sup>b</sup>	Number Animals Dead <sup>b</sup>
Preimmune <sup>1</sup>	0	5
CTB <sup>1</sup> : 400 µg	5	0
CTB (AP on pPB1750-2360): <sup>2</sup> 875 µg	5	0
CTB (AP on pMB1750-1970): <sup>2</sup> 875 µg	5	0
CTB (AP on pMB1970-2360): <sup>2</sup> 500 µg	5	0

*C. difficile* toxin B (CTB) (Tech Lab: at 5 µg/ml, 25 µg total) at lethal concentration to hamsters is added to antibody and incubated for one hour at 37°C. After incubation this mixture is injected intraperitoneally (IP) into hamsters. Each treatment group received toxin premixed with antibody raised against the indicated protein, as either: 1) 4X antibody PEG prep or 2) affinity purified (AP) antibody (from CTB PEG prep, on indicated columns). The amount of specific antibody in each prep is indicated; the amount is directly determined for affinity purified preps and is estimated for the 4X CTB as described in Example 15.

<sup>a</sup> The numbers in each group represent numbers of hamsters dead or alive, 2 hr post IP administration of toxin/antibody mixture.

The observation that antibodies affinity purified against the non-overlapping pMB1750-1970 and pMB1970-2360 proteins neutralized toxin B raised the possibility that either 1) antibodies specific to repeat sub-regions are sufficient to neutralize toxin B or 2) sub-region specific proteins can bind most or all repeat specific antibodies present in the CTB polyclonal pool. This would likely be due to conformational similarities between repeats, since homology in the primary amino acid sequences between different repeats is in the range of only 25-75% [Eichel-Streiber, *et al.* (1992) Molec. Gen. Genetics 233:260]. These possibilities were tested by affinity chromatography.

The CTB PEG prep was sequentially depleted 2X on the pMB1750-1970 column: only a small elution peak was observed after the second chromatography, indicating that most reactive antibodies were removed. This interval depleted CTB preparation was then chromatographed on the pPB1850-2360 column: no antibody bound to the column. The reactivity of the CTB and CTB (pMB1750-1970 depleted) preps to pPB1750-2360, pPB1850-2360, pMB1750-1970 and pMB1970-2360 proteins was then determined by ELISA using the protocol described in Example 13(c). Briefly, 96-well microtiter plates (Falcon, Pro-Bind Assay Plates) were coated with recombinant protein by adding 100 µl volumes of protein at 1-2 µg/ml in PBS containing 0.005% thimerosal to each well and incubating overnight at 4°C. The next morning, the coating suspensions were decanted and the wells were washed three

times using PBS. In order to block non-specific binding sites, 100  $\mu$ l of 1.0% BSA (Sigma) in PBS (blocking solution) was then added to each well, and the plates were incubated for 1 hr. at 37°C. The blocking solution was decanted and duplicate samples of 150  $\mu$ l of diluted antibody was added to the first well of a dilution series. The initial testing serum dilution was (1/200 for CTB prep. (the concentration of depleted CTB was standardized by OD<sub>280</sub>) in blocking solution containing 0.5% Tween 20, followed by 5-fold serial dilutions into this solution. This was accomplished by serially transferring 30  $\mu$ l aliquots to 120  $\mu$ l buffer, mixing, and repeating the dilution into a fresh well. After the final dilution, 30  $\mu$ l was removed from the well such that all wells contained 120  $\mu$ l final volume. A total of 5 such dilutions were performed (4 wells total). The plates were incubated for 1 hr at 37°C. Following this incubation, the serially diluted samples were decanted and the wells were washed three times using PBS containing 0.5% Tween 20 (PBST), followed by two 5 min washes using BBS-Tween and a final three washes using PBST. To each well, 100  $\mu$ l of 1:1000 diluted secondary antibody [rabbit anti-chicken IgG alkaline phosphatase (Sigma) diluted in blocking solution containing 0.5% Tween 20] was added, and the plate was incubated 1 hr at 37°C. The conjugate solutions were decanted and the plates were washed 6 times in PBST, then once in 50 mM Na<sub>2</sub>CO<sub>3</sub>, 10 mM MgCl<sub>2</sub>, pH 9.5. The plates were developed by the addition of 100  $\mu$ l of a solution containing 1 mg/ml para-nitro phenyl phosphate (Sigma) dissolved in 50 mM Na<sub>2</sub>CO<sub>3</sub>, 10 mM MgCl<sub>2</sub>, pH9.5 to each well. The plates were then incubated at room temperature in the dark for 5-45 min. The absorbency of each well was measured at 410 nm using a Dynatech MR 700 plate reader.

As predicted by the affinity chromatography results, depletion of the CTB prep on the pMB1750-1970 column removed all detectable reactivity to the pMB1970-2360 protein. The reciprocal purification of a CTB prep that was depleted on the pMB1970-2360 column yielded no bound antibody when chromatographed on the pMB1750-1970 column. These results demonstrate that all repeat reactive antibodies in the CTB polyclonal pool recognize a conserved structure that is present in non-overlapping repeats. Although it is possible that this conserved structure represents rare conserved linear epitopes, it appears more likely that the neutralizing antibodies recognize a specific protein conformation. This conclusion was also suggested by the results of Western blot hybridization analysis of CTB reactivity to these recombinant proteins.

Western blots of 7.5% SDS-PAGE gels, loaded and electrophoresed with defined quantities of each recombinant protein, were probed with the CTB polyclonal antibody

preparation. The blots were prepared and developed with alkaline phosphatase as described in Example 3. The results are shown in Figure 24.

Figure 24 depicts a comparison of immunoreactivity of IgY antibody raised against either native or recombinant toxin B antigen. Equal amounts of pMB1750-1970 (lane 1), pMB1970-2360 (lane 2), pPB1850-2360 (lane 3) as well as a serial dilution of pPB1750-2360 (lanes 4-6 comprising 1X, 1/10X and 1/100X amounts, respectively) proteins were loaded in duplicate and resolved on a 7.5% SDS-PAGE gel. The gel was blotted and each half was hybridized with PEG prep IgY antibodies from chickens immunized with either native CTB or pPB1750-2360 protein. Note that the full-length pMB1750-1970 protein was identified only by antibodies reactive to the recombinant protein (arrows).

Although the CTB prep reacts with the pPB1750-2360, pPB1850-2360, and pMB1970-2360 proteins, no reactivity to the pMB1750-1970 protein was observed (Figure 24). Given that all repeat reactive antibodies can be bound by this protein during affinity chromatography, this result indicates that the protein cannot fold properly on Western blots. Since this eliminates all antibody reactivity, it is unlikely that the repeat reactive antibodies in the CTB prep recognize linear epitopes. This may indicate that in order to induce protective antibodies, recombinant toxin B protein will need to be properly folded.

### **c) Generation And Evaluation Of Antibodies Reactive To Recombinant Toxin B Polypeptides**

#### **i) Generation Of Antibodies Reactive To Recombinant Toxin B Proteins**

Antibodies against recombinant proteins were generated in egg laying Leghorn hens as described in Example 13. Antibodies were raised [using Freund's adjuvant (Gibco) unless otherwise indicated] against the following recombinant proteins: 1) a mixture of Interval 1+2 proteins (see Figure 18); 2) a mixture of interval 4 and 5 proteins (see Figure 18); 3) pMB1970-2360 protein; 4) pPB1750-2360 protein; 5) pMB1750-2360; 6) pMB1750-2360 [Titermax adjuvant (Vaxcell)]; 7) pMB1750-2360 [Gerbu adjuvant (Biotech)]; 8) pMBp1750-2360 protein; 9) pPB1850-2360; and 10) pMB1850-2360.

Chickens were boosted at least 3 times with recombinant protein until ELISA reactivity [using the protocol described in b) above with the exception that the plates were coated with pPB1750-2360 protein] of polyclonal PEG preps was at least equal to that of the CTB polyclonal antibody PEG prep. ELISA titers were determined for the PEG preps from

all of the above immunogens and were found to be comparable ranging from 1:12500 to 1:62500. High titers were achieved in all cases except in 6) pMB1750-2360 in which strong titers were not observed using the Titermax adjuvant, and this preparation was not tested further.

5

ii) **Evaluation Of Antibodies Reactive To  
Recombinant Proteins By Western Blot  
Hybridization**

10 Western blots of 7.5% SDS-PAGE gels, loaded and electrophoresed with defined quantities of recombinant protein (pMB1750-1970, pPB1850-2360, and pMB1970-2360 proteins and a serial dilution of the pPB1750-2360 to allow quantification of reactivity), were probed with the CTB, pPB1750-2360, pMB1750-2360 and pMB1970-2360 polyclonal antibody preparations (from chickens immunized using Freund's adjuvant). The blots were prepared and developed with alkaline phosphatase as described above in b).

15 As shown in Figure 24, the CTB and pMB1970-2360 preps reacted strongly with the pPB1750-2360, pPB1850-2360, and pMB1970-2360 proteins while the pPB1750-2360 and pMB1970-2360 (Gerbu) preparations reacted strongly with all four proteins. The Western blot reactivity of the pPB1750-2360 and pMB1970-2360 (Gerbu) preparations were equivalent to that of the CTB preparation, while reactivity of the pMB1970-2360 preparation was <10% that of the CTB prep. Despite equivalent ELISA reactivities only weak reactivity (approximately 1%) to the recombinant proteins were observed in PEG preps from two independent groups immunized with the pMB1750-2360 protein and one group immunized with the pMB1750-2360 preparation using Freund's adjuvant.

25 Affinity purification was utilized to determine if this difference in immunoreactivity by Western blot analysis reflects differing antibody titers. Fifty ml 2X PEG preparations from chickens immunized with either pMB1750-2360 or pMB1970-2360 protein were chromatographed on the pPB1750-2360 affinity column from b) above, as described. The yield of affinity purified antibody (% total protein in preparation) was equivalent to the yield obtained from a CTB PEG preparation in b) above. Thus, differences in Western reactivity reflect a qualitative difference in the antibody pools, rather than quantitative differences..

30 These results demonstrate that certain recombinant proteins are more effective at generating high affinity antibodies (as assayed by Western blot hybridization).

iii) ***In Vivo* Neutralization Of Toxin B Using  
Antibodies Reactive To Recombinant Protein**

The *in vivo* hamster model [described in Examples 9 and 14(b)] was utilized to assess the neutralizing ability of antibodies raised against recombinant toxin B proteins. The results from three experiments are shown below in Tables 27-29.

The ability of each immunogen to neutralize toxin B *in vivo* has been compiled and is shown in Table 30. As predicted from the recombinant protein-CTB premix studies (Table 24) only antibodies to Interval 3 (1750-2366) and not the other regions of toxin B (*i.e.*, intervals 1-5) are protective. Unexpectedly, antibodies generated to INT-3 region expressed in pMAL vector (pMB1750-2360 and pMpB1750-2360) using Freund's adjuvant were non-neutralizing. This observation is reproducible, since no neutralization was observed in two independent immunizations with pMB1750-2360 and one immunization with pMpB1750-2360. The fact that 5X quantities of affinity purified toxin B repeat specific antibodies from pMB1750-2360 PEG preps cannot neutralize toxin B while 1X quantities of affinity purified anti-CTB antibodies can (Table 28) demonstrates that the differential ability of CTB antibodies to neutralize toxin B is due to qualitative rather than quantitative differences in these antibody preparations. Only when this region was expressed in an alternative vector (pPB1750-2360) or using an alternative adjuvant with the pMB1750-2360 protein were neutralizing antibodies generated. Importantly, antibodies raised using Freund's adjuvant to pPB1850-2360, which contains a fragment that is only 100 amino acids smaller than recombinant pPB1750-2360, are unable to neutralize toxin B *in vivo* (Table 27); note also that the same vector is used for both pPB1850-2360 and pPB1750-2360.

TABLE 27

*In Vivo* Neutralization Of Toxin B

Treatment Group <sup>a</sup>	Number Animals Alive <sup>b</sup>	Number Animals Dead <sup>b</sup>
Preimmune	0	5
CTB	5	0
INT1+2	0	5
INT 4+5	0	5
pMB1750-2360	0	5
pMB1970-2360	0	5
pPB1750-2360	5	0

*C. difficile* toxin B (CTB) (at 5 µg/ml; 25 µg total; Tech Lab) at lethal concentration to hamsters is added to antibody and incubated for one hour at 37°C. After incubation this mixture is injected intraperitoneally (IP) into hamsters. Each treatment group received toxin premixed with antibody raised against the indicated protein, as a 4X antibody PEG prep.

The numbers in each group represent numbers of hamsters dead or alive, 2 hours post IP administration of toxin/antibody mixture.

TABLE 28

*In Vivo* Neutralization Of Toxin B Using Affinity Purified Antibodies

Treatment Group <sup>a</sup>	Number Animals Alive <sup>b</sup>	Number Animals Dead <sup>b</sup>
Preimmune(1)	0	5
CTB(1)	5	0
pPB1750-2360(1)	5	0
1.5 mg anti-pMB1750-2360(2)	1	4
1.5 mg anti-pMB1970-2360(2)	0	5
300 µg anti-CTB(2)	5	0

*C. difficile* toxin B (CTB) (at 5 µg/ml; 25 µg total; Tech Lab) at lethal concentration to hamsters is added to antibody and incubated for one hour at 37°C. After incubation, 1 ml of this mixture is injected intraperitoneally (IP) into hamsters. Each treatment group received toxin premixed with antibody raised against the indicated protein, as either (1) 4X antibody PEG prep or (2) affinity purified antibody (on a pPB1750-2360 resin), either 1.5 mg/group (anti-pMB1750-2360 and anti-pMB1970-2360; used undiluted affinity purified antibody) or 350 µg/group (anti-CTB, repeat specific; used 1/5 diluted anti-CTB antibody).

The numbers in each group represent numbers of hamsters dead or alive, 2 hr post-IP administration of toxin/antibody mixture.

TABLE 29

Generation Of Neutralizing Antibodies Utilizing The Gerbu Adjuvant

Treatment Group <sup>a</sup>	Number Animals Alive <sup>b</sup>	Number Animals Dead <sup>b</sup>
Preimmune	0	5
CTB	5	0
pMB1970-2360	0	5
pMB1850-2360	0	5
pPB1850-2360	0	5
pMB1750-2360 (Gerbu adj)	5	0

*C. difficile* toxin B (CTB) (Tech Lab) at lethal concentration to hamsters is added to antibody and incubated for one hour at 37°C. After incubation this mixture is injected intraperitoneally (IP) into hamsters. Each treatment group received toxin premixed with antibody raised against the indicated protein, as a 4X antibody PEG prep.

The numbers in each group represent numbers of hamsters dead or alive, 2hrs post IP administration of toxin-antibody mixture.



TABLE 30

*In Vivo* Neutralization Of Toxin B

Immunogen	Adjuvant	Tested Preparation <sup>a</sup>	Antigen Utilized For AP	<i>In vivo</i> Neutralization <sup>b</sup>
Preimmune	NA <sup>c</sup>	PEG	NA	no
CTB (native)	Titermax	PEG	NA	yes
CTB (native)	Titermax	AP	pPB1750-2360	yes
CTB (native)	Titermax	AP	pPB1850-2360	yes
CTB (native)	Titermax	AP	pPB1750-1970	yes
CTB (native)	Titermax	AP	pPB1970-2360	yes
pMB1750-2360	Freunds	PEG	NA	no
pMB1750-2360	Freunds	AP	pPB1750-2360	no
pMB1750-2360	Gerbu	PEG	NA	yes
pMB1970-2360	Freunds	PEG	NA	no
pMB1970-2360	Freunds	AP	pPB1750-2360	no
pPB1750-2360	Freunds	PEG	NA	yes
pPB1850-2360	Freunds	PEG	NA	no
pMB1850-2360	Freunds	PEG	NA	no
INT 1-2	Freunds	PEG	NA	no
INT 4-5	Freunds	PEG	NA	no

<sup>a</sup> Either PEG preparation (PEG) or affinity purified antibodies (AP).

<sup>b</sup> 'Yes' denotes complete neutralization (0/5 dead) while 'no' denotes no neutralization (5/5 dead) of toxin B, 2 hours post-administration of mixture.

<sup>c</sup> 'NA' denotes not applicable.

The pPB1750-2360 antibody pool confers significant *in vivo* protection, equivalent to that obtained with the affinity purified CTB antibodies. This correlates with the observed high affinity of this antibody pool (relative to the pMB1750-2360 or pMB1970-2360 pools) as assayed by Western blot analysis (Figure 24). These results provide the first demonstration that *in vivo* neutralizing antibodies can be induced using recombinant toxin B protein as immunogen.

The failure of high concentrations of antibodies raised against the pMB1750-2360 protein (using Freunds adjuvant) to neutralize, while the use of Gerbu adjuvant and pMB1750-2360 protein generates a neutralizing response, demonstrates that conformation or presentation of this protein is essential for the induction of neutralizing antibodies. These

results are consistent with the observation that the neutralizing antibodies produced when native CTB is used as an immunogen appear to recognize conformational epitopes [see section b) above]. This is the first demonstration that the conformation or presentation of recombinant toxin B protein is essential to generate high titers of neutralizing antibodies.

## EXAMPLE 20

### Determination Of Quantitative And Qualitative Differences Between pMB1750-2360, pMB1750-2360 (Gerbu) Or pPB1750-2360 IgY Polyclonal Antibody Preparations

In Example 19, it was demonstrated that toxin B neutralizing antibodies could be generated using specific recombinant toxin B proteins (pPB1750-2360) or specific adjuvants. Antibodies raised against pMB1750-2360 were capable of neutralizing the enterotoxin effect of toxin B when the recombinant protein was used to immunize hens in conjunction with the Gerbu adjuvant, but not when Freund's adjuvant was used. To determine the basis for these antigen and adjuvant restrictions, toxin B-specific antibodies present in the neutralizing and non-neutralizing PEG preparations were isolated by affinity chromatography and tested for qualitative or quantitative differences. The example involved a) purification of anti-toxin B specific antibodies from pMB1750-2360 and pPB1750-2360 PEG preparations and b) *in vivo* neutralization of toxin B using the affinity purified antibody.

#### a) Purification Of specific Antibodies From pMB1750-2360 And pPB1750-2360 PEG Preparations

To purify and determine the concentration of specific antibodies (expressed as the percent of total antibody) within the pPB1750-2360 (Freund's and Gerbu) and pPB1750-2360 PEG preparations, defined quantities of these antibody preparations were chromatographed on an affinity column containing the entire toxin B repeat region (pPB1750-2360). The amount of affinity purified antibody was then quantified.

An affinity column containing the recombinant toxin B repeat protein, pPB1750-2360, was made as follows. Four ml of PBS-washed Actigel resin (Sterogene) was coupled with 5 mg of pPB1750-2360 affinity purified protein (dialyzed into PBS; estimated to be greater than 95% full length fusion protein) in a 15 ml tube (Falcon) containing 1/10 final volume Ald-coupling solution (1M sodium cyanoborohydride). Aliquots of the supernatant from the

coupling reactions, before and after coupling, were assessed by Coomassie staining of 7.5% SDS-PAGE gels. Based on protein band intensities, greater than 95% (approximately 5 mg) of recombinant protein was coupled to the resin. The coupled resin was poured into a 10 ml column (BioRad), washed extensively with PBS, pre-eluted with 4M guanidine-HCl (in 10 mM Tris-HCl, pH 8.0; 0.005% thimerosal) and re-equilibrated in PBS and stored at 4°C.

Aliquots of pMB1750-2360, pMB1750-2360 (Gerbu) or pPB1750-2360 IgY polyclonal antibody preparations (PEG preps) were affinity purified on the above column as follows.

The column was attached to an UV monitor (ISCO), and washed with PBS. Forty ml aliquots of 2X PEG preps (filter sterilized using a 0.45  $\mu$  filter and quantified by OD<sub>280</sub> before

chromatography) was applied. The column was washed with PBS until the baseline was re-established (the column flow-through was saved), washed with BBSTween to elute

nonspecifically binding antibodies and re-equilibrated with PBS. Bound antibody was eluted from the column in 4M guanidine-HCl (in 10 mM Tris-HCl, pH 8.0, 0.005% thimerosal) and the entire elution peak collected in a 15 ml tube (Falcon). The column was re-equilibrated,

and the column eluate re-chromatographed as described above. The antibody preparations were quantified by UV absorbance (the elution buffer was used to zero the spectrophotometer). Approximately 10 fold higher concentrations of total purified antibody was obtained upon elution of the first chromatography pass relative to the second pass. The low yield from the second chromatography pass indicated that most of the specific antibodies were removed by the first round of chromatography.

Pools of affinity purified specific antibodies were prepared by dialysis of the column elutes after the first column chromatography pass for the pMB1750-2360, pMB1750-2360 (Gerbu) or pPB1750-2360 IgY polyclonal antibody preparations. The elutes were collected on ice and immediately dialyzed against a 100-fold volume of PBS at 4°C for 2 hrs. The samples were then dialyzed against 3 changes of a 65-fold volume of PBS at 4°C. Dialysis was performed for a minimum of 8 hrs per change of PBS. The dialyzed samples were collected, centrifuged to remove insoluble debris, quantified by OD<sub>280</sub>, and stored at 4°C.

The percentage of toxin B repeat-specific antibodies present in each preparation was determined using the quantifications of antibody yields from the first column pass (amount of specific antibody recovered after first pass/total protein loaded). The yield of repeat-specific affinity purified antibody (expressed as the percent of total protein in the preparation) in: 1) the pMB1750-2360 PEG prep was approximately 0.5%, 2) the pMB1750-2360 (Gerbu) prep was approximately 2.3%, and 3) the pPB1750-2360 prep was approximately 0.4%.

Purification of a CTB IgY polyclonal antibody preparation on the same column demonstrated that the concentration of toxin B repeat specific antibodies in the CTB preparation was 0.35%.

These results demonstrate that 1) the use of Gerbu adjuvant enhanced the titer of specific antibody produced against the pMB1750-2360 protein 5-fold relative to immunization using Freund's adjuvant, and 2) the differences seen in the *in vivo* neutralization ability of the pMB1750-2360 (not neutralizing) and pPB1750-2360 (neutralizing) and CTB (neutralizing) PEG preps seen in Example 19 was not due to differences in the titers of repeat-specific antibodies in the three preparations because the titer of repeat-specific antibody was similar for all three preps; therefore the differing ability of the three antibody preparations to neutralize toxin B must reflect qualitative differences in the induced toxin B repeat-specific antibodies. To confirm that qualitative differences exist between antibodies raised in hens immunized with different recombinant proteins and/or different adjuvants, the same amount of affinity purified anti-toxin B repeat (aa 1870-2360 of toxin B) antibodies from the different preparations was administered to hamsters using the *in vivo* hamster model as described below.

**b) *In vivo* Neutralization Of Toxin B Using Affinity Purified Antibody**

The *in vivo* hamster model was utilized to assess the neutralizing ability of the affinity purified antibodies raised against recombinant toxin B proteins purified in (a) above. As well, a 4X IgY PEG preparation from a second independent immunization utilizing the pPB1750-2360 antigen with Freund's adjuvant was tested for *in vivo* neutralization. The results are shown in Table 31.

The results shown in Table 31 demonstrate that:

- 1) as shown in Example 19 and reproduced here, 1.5 mg of affinity purified antibody from pMB1750-2360 immunized hens using Freund's adjuvant does not neutralize toxin B *in vivo*. However, 300 µg of affinity purified antibody from similarly immunized hens utilizing Gerbu adjuvant demonstrated complete neutralization of toxin B *in vivo*. This demonstrates that Gerbu adjuvant, in addition to enhancing the titer of antibodies reactive to the pMB1750-2360 antigen relative to Freund's adjuvant (demonstrated in (a) above), also enhances the yield of neutralizing antibodies to this antigen, greater than 5 fold.

- 2) Complete *in vivo* neutralization of toxin B was observed with 1.5 mg of affinity purified antibody from hens immunized with pPB1750-2360 antigen, but not with pMB1750-2360 antigen, when Freund's adjuvant was used. This demonstrates, using standardized toxin B repeat-specific antibody concentrations, that neutralizing antibodies were induced when pPB1750-2360 but not pMB1750-2360 was used as the antigen with Freund's adjuvant.
- 3) Complete *in vivo* neutralization was observed with 300 µg of pMB1750-2360 (Gerbu) antibody, but not with 300 µg of pPB1750-2360 (Freund's) antibody. Thus the pMB1750-2360 (Gerbu) antibody has a higher titer of neutralizing antibodies than the pPB1750-2360 (Freund's) antibody.
- 4) Complete neutralization of toxin B was observed using 300 µg of CTB antibody [affinity purified (AP)] but not 100 µg CTB antibody (AP or PEG prep). This demonstrates that greater than 100 µg of toxin B repeat-specific antibody (anti-CTB) is necessary to neutralize 25 µg toxin B *in vivo* in this assay, and that affinity purified antibodies specific to the toxin B repeat interval neutralize toxin B as effectively as the PEP prep of IgY raised against the entire CTB protein (shown in this assay).
- 5) As was observed with the initial pPB1750-2360 (IgY) PEG preparation (Example 19), complete neutralization was observed with a IgY PEG preparation isolated from a second independent group of pPB1750-2360 (Freund's) immunized hens. This demonstrates that neutralizing antibodies are reproducibly produced when hens are immunized with pPB1750-2360 protein utilizing Freund's adjuvant.

TABLE 31

*In vivo* Neutralization Of Toxin B Using Affinity Purified Antibodies

Treatment Group <sup>a</sup>	Number Animals Alive <sup>b</sup>	Number Animals Dead <sup>b</sup>
Preimmune <sup>1</sup>	0	5
CTB (300 µg) <sup>2</sup>	5	0
CTB (100 µg) <sup>2</sup>	1	4
pMB1750-2360 (G) (5 mg) <sup>2</sup>	5	0
pMB1750-2360 (G) (1.5 mg) <sup>2</sup>	5	0
pMB1750-2360 (G) (300 µg) <sup>2</sup>	5	0
pMB1750-2360 (F) (1.5 mg) <sup>2</sup>	0	5
pPB1750-2360 (F) (1.5 mg) <sup>2</sup>	5	0
pPB1750-2360 (F) (300 µg) <sup>2</sup>	1	4
CTB (100 µg) <sup>2</sup>	2	3
pPB1750-2360 (F) (500 µg) <sup>2</sup>	5	0

*C. difficile* toxin B (CTB) (Tech Lab) at lethal concentration to hamsters (25 µg) was added to the antibody (amount of specific antibody is indicated) and incubated for one hour at 37°C. After incubation, this mixture was injected IP into hamsters (1.5 total mix injected per hamster). Each treatment group received toxin premixed with antibody raised against the indicated protein (G=gerbu adjuvant, F=Freunds adjuvant). <sup>1</sup> indicates the antibody was a 4X IgY PEG prep; <sup>2</sup> indicates the antibody was affinity purified on a pPB1750-2360 resin; and <sup>3</sup> indicates that the antibody was a 1X IgY PEG prep.

The numbers in each group represent numbers of hamsters dead or alive, 2 hrs post IP administration of toxin:antibody mixture.

## EXAMPLE 21

Diagnostic Enzyme Immunoassays For *C. difficile* Toxins A And B

The ability of the recombinant toxin proteins and antibodies raised against these recombinant proteins (described in the above examples) to form the basis of diagnostic assays for the detection of clostridial toxin in a sample was examined. Two immunoassay formats were tested to quantitatively detect *C. difficile* toxin A and toxin B from a biological specimen. The first format involved a competitive assay in which a fixed amount of recombinant toxin A or B was immobilized on a solid support (e.g., microtiter plate wells) followed by the addition of a toxin-containing biological specimen mixed with affinity-purified or PEG fractionated antibodies against recombinant toxin A or B. If toxin is present in a specimen, this toxin will compete with the immobilized recombinant toxin protein for

binding to the anti-recombinant antibody thereby reducing the signal obtained following the addition of a reporter reagent. The reporter reagent detects the presence of antibody bound to the immobilized toxin protein.

In the second format, a sandwich immunoassay was developed using affinity-purified antibodies to recombinant toxin A and B. The affinity-purified antibodies to recombinant toxin A and B were used to coat microtiter wells instead of the recombinant polypeptides (as was done in the competitive assay format). Biological samples containing toxin A or B were then added to the wells followed by the addition of a reporter reagent to detect the presence of bound toxin in the well.

a) **Competitive Immunoassay For The Detection Of *C. difficile* Toxin**

Recombinant toxin A or B was attached to a solid support by coating 96 well microtiter plates with the toxin protein at a concentration of 1 µg/ml in PBS. The plates were incubated overnight at 2-8°C. The following morning, the coating solutions were removed and the remaining protein binding sites on the wells were blocked by filling each well with a PBS solution containing 0.5% BSA and 0.05% Tween-20. Native *C. difficile* toxin A or B (Tech Lab) was diluted to 4 µg/ml in stool extracts from healthy Syrian hamsters (Sasco). The stool extracts were made by placing fecal pellets in a 15 ml centrifuge tube; PBS was added at 2 ml/pellet and the tube was vortexed to create a uniform suspension. The tube was then centrifuged at 2000 rpm for 5 min at room temperature. The supernatant was removed; this comprises the stool extract. Fifty µl of the hamster stool extract was pipetted into each well of the microtiter plates to serve as the diluent for serial dilutions of the 4 µg/ml toxin samples. One hundred µl of the toxin samples at 4 µg/ml was pipetted into the first row of wells in the microtiter plate, and 50 µl aliquots were removed and diluted serially down the plate in duplicate. An equal volume of affinity purified anti-recombinant toxin antibodies [1 ng/well of anti-pMA1870-2680 antibody was used for the detection of toxin A; 0.5 ng/well of anti-pMB1750-2360(Gierbu) was used for the detection of toxin B] were added to appropriate wells, and the plates were incubated at room temperature for 2 hours with gentle agitation. Wells serving as negative control contained antibody but no native toxin to compete for binding.

Unbound toxin and antibody were removed by washing the plates 3 to 5 times with PBS containing 0.05% Tween-20. Following the wash step, 100 µl of rabbit anti-chicken IgG

antibody conjugated to alkaline phosphatase (Sigma) was added to each well and the plates were incubated for 2 hours at room temperature. The plates were then washed as before to remove unbound secondary antibody. Freshly prepared alkaline phosphatase substrate (1 mg/ml p-nitrophenyl phosphate (Sigma) in 50 mM  $\text{Na}_2\text{CO}_3$ , pH 9.5; 10 mM  $\text{MgCl}_2$ ) was added to each well. Once sufficient color developed, the plates were read on a Dynatech MR700 microtiter plate reader using a 410 nm filter.

The results are summarized in Tables 32 and 33. For the results shown in Table 32, the wells were coated with recombinant toxin A protein (pMA1870-2680). The amount of native toxin A added (present as an addition to solubilized hamster stool) to a given well is indicated (0 to 200 ng). Antibody raised against the recombinant toxin A protein, pMA1870-2680, was affinity purified on the an affinity column containing pPA1870-2680 (described in Example 20). As shown in Table 32, the recombinant toxin A protein and affinity-purified antitoxin can be used for the basis of a competitive immunoassay for the detection of toxin A in biological samples.

Similar results were obtained using the recombinant toxin B, pPB1750-2360, and antibodies raised against pMB1750-2360(Gerbu). For the results shown in Table 33, the wells were coated with recombinant toxin B protein (pPB1750-2360). The amount of native toxin B added (present as an addition to solubilized hamster stool) to a given well is indicated (0 to 200 ng). Antibody raised against the recombinant toxin B protein, pMB1750-2360(Gerbu), was affinity purified on the an affinity column containing pPB1850-2360 (described in Example 20). As shown in Table 33, the recombinant toxin B protein and affinity-purified antitoxin can be used for the basis of a competitive immunoassay for the detection of toxin B in biological samples.

In this competition assay, the reduction is considered significant over the background levels at all points; therefore the assay can be used to detect samples containing less than 12.5 ng toxin A/well and as little as 50-100 ng toxin B/well.



TABLE 32

Competitive Inhibition Of Anti-*C. difficile* Toxin A By Native Toxin A

ng Toxin A/Well	OD <sub>410</sub> Readout
200	0.176
100	0.253
50	0.240
25	0.259
12.5	0.309
6.25	0.367
3.125	0.417
0	0.590

TABLE 33

Competitive Inhibition Of Anti-*C. difficile* Toxin B By Native Toxin B

ng Toxin B/Well	OD <sub>410</sub> Readout
200	0.392
100	0.566
50	0.607
25	0.778
12.5	0.970
6.25	0.902
3.125	1.040
0	1.055

These competitive inhibition assays demonstrate that native *C. difficile* toxins and recombinant *C. difficile* toxin proteins can compete for binding to antibodies raised against recombinant *C. difficile* toxins demonstrating that these anti-recombinant toxin antibodies provide effective diagnostic reagents.

#### b) Sandwich Immunoassay For The Detection Of *C. difficile* Toxin

Affinity-purified antibodies against recombinant toxin A or toxin B were immobilized to 96 well microtiter plates as follows. The wells were passively coated overnight at 4°C with affinity purified antibodies raised against either pMA1870-2680 (toxin A) or pMB1750-

2360(Gerbu) (toxin B). The antibodies were affinity purified as described in Example 20. The antibodies were used at a concentration of 1 µg/ml and 100 µl was added to each microtiter well. The wells were then blocked with 200 µl of 0.5% BSA in PBS for 2 hours at room temperature and the blocking solution was then decanted. Stool samples from healthy Syrian hamsters were resuspended in PBS, pH 7.4 (2 ml PBS/stool pellet was used to resuspend the pellets and the sample was centrifuged as described above). The stool suspension was then spiked with native *C. difficile* toxin A or B (Tech Lab) at 4 µg/ml. The stool suspensions containing toxin (either toxin A or toxin B) were then serially diluted two-fold in stool suspension without toxin and 50 µl was added in duplicate to the coated microtiter wells. Wells containing stool suspension without toxin served as the negative control.

The plates were incubated for 2 hours at room temperature and then were washed three times with PBS. One hundred µl of either goat anti-native toxin A or goat anti-native toxin B (Tech Lab) diluted 1:1000 in PBS containing 1% BSA and 0.05% Tween 20 was added to each well. The plates were incubated for another 2 hours at room temperature. The plates were then washed as before and 100 µl of alkaline phosphatase-conjugated rabbit anti-goat IgG (Cappel, Durham, N.C.) was added at a dilution of 1:1000. The plates were incubated for another 2 hours at room temperature. The plates were washed as before then developed by the addition of 100 µl/well of a substrate solution containing 1 mg/ml p-nitrophenyl phosphate (Sigma) in 50 mM Na<sub>2</sub>CO<sub>3</sub>, pH 9.5; 10 mM MgCl<sub>2</sub>. The absorbance of each well was measured using a plate reader (Dynatech) at 410 nm. The assay results are shown in Tables 34 and 35.

TABLE 34

*C. difficile* Toxin A Detection In Stool Using Affinity-Purified Antibodies Against Toxin A

ng Toxin A/Well	OD <sub>410</sub> Readout
200	0.9
100	0.8
50	0.73
25	0.71
12.5	0.59
6.25	0.421
0	0

TABLE 35

*C. difficile* Toxin B Detection In Stool Using Affinity-Purified Antibodies Against Toxin B

ng Toxin B/Well	OD <sub>410</sub> Readout
200	1.2
100	0.973
50	0.887
25	0.846
12.5	0.651
6.25	0.431
0	0.004

The results shown in Tables 34 and 35 show that antibodies raised against recombinant toxin A and toxin B fragments can be used to detect the presence of *C. difficile* toxin in stool samples. These antibodies form the basis for a sensitive sandwich immunoassay which is capable of detecting as little as 6.25 ng of either toxin A or B in a 50 µl stool sample. As shown above in Tables 34 and 35, the background for this sandwich immunoassay is extremely low; therefore, the sensitivity of this assay is much lower than 6.25 ng toxin/well. It is likely that toxin levels of 0.5 to 1.0 pg/well could be detected by this assay.

The results shown above in Tables 32-35 demonstrate clear utility of the recombinant reagents in *C. difficile* toxin detection systems.

## EXAMPLE 22

### Construction And Expression Of *C. botulinum* C Fragment Fusion Proteins

The *C. botulinum* type A neurotoxin gene has been cloned and sequenced [Thompson, *et al.*, Eur. J. Biochem. 189:73 (1990)]. The nucleotide sequence of the toxin gene is available from the EMBL/GenBank sequence data banks under the accession number X52066; the nucleotide sequence of the coding region is listed in SEQ ID NO:27. The amino acid sequence of the *C. botulinum* type A neurotoxin is listed in SEQ ID NO:28. The type A neurotoxin gene is synthesized as a single polypeptide chain which is processed to form a dimer composed of a light and a heavy chain linked via disulfide bonds. The 50 kD carboxy-terminal portion of the heavy chain is referred to as the C fragment or the H<sub>c</sub> domain.

Previous attempts by others to express polypeptides comprising the C fragment of *C. botulinum* type A toxin as a native polypeptide (*e.g.*, not as a fusion protein) in *E. coli* have

been unsuccessful [H.F. LaPenotiere, *et al.* in *Botulinum and Tetanus Neurotoxins*, DasGupta, Ed., Plenum Press, New York (1993), pp. 463-466]. Expression of the C fragment as a fusion with the *E. coli* MBP was reported to result in the production of insoluble protein (H.F. LaPenotiere, *et al.*, *supra*).

In order to produce soluble recombinant C fragment proteins in *E. coli*, fusion proteins comprising a synthetic C fragment gene derived from the *C. botulinum* type A toxin and either a portion of the *C. difficile* toxin protein or the MBP were constructed. This example involved a) the construction of plasmids encoding C fragment fusion proteins and b) expression of *C. botulinum* C fragment fusion proteins in *E. coli*.

#### a) Construction Of Plasmids Encoding C Fragment Fusion Proteins

In Example 11, it was demonstrated that the *C. difficile* toxin A repeat domain can be efficiently expressed and purified in *E. coli* as either native (expressed in the pET 23a vector in clone pPA1870-2680) or fusion (expressed in the pMALc vector as a fusion with the *E. coli* MBP in clone pMA1870-2680) proteins. Fusion proteins comprising a fusion between the MBP, portions of the *C. difficile* toxin A repeat domain (shown to be expressed as a soluble fusion protein) and the C fragment of the *C. botulinum* type A toxin were constructed. A fusion protein comprising the C fragment of the *C. botulinum* type A toxin and the MBP was also constructed.

Figure 25 provides a schematic representation of the botulinal fusion proteins along with the donor constructs containing the *C. difficile* toxin A sequences or *C. botulinum* C fragment sequences which were used to generate the botulinal fusion proteins. In Figure 25, the solid boxes represent *C. difficile* toxin A gene sequences, the open boxes represent *C. botulinum* C fragment sequences and the solid black ovals represent the *E. coli* MBP. When the name for a restriction enzyme appears inside parenthesis, this indicates that the restriction site was destroyed during construction. An asterisk appearing with the name for a restriction enzyme indicates that this restriction site was recreated at the cloning junction.

In Figure 25, a restriction map of the pMA1870-2680 and pPA1100-2680 constructs (described in Example 11) which contain sequences derived from the *C. difficile* toxin A repeat domain are shown; these constructs were used as the source of *C. difficile* toxin A gene sequences for the construction of plasmids encoding fusions between the *C. botulinum* C fragment gene and the *C. difficile* toxin A gene. The pMA1870-2680 expression construct

expresses high levels of soluble, intact fusion protein (20 mg/liter culture) which can be affinity purified on an amylose column (purification described in Example 11d).

The pAlterBot construct (Figure 25) was used as the source of *C. botulinum* C fragment gene sequences for the botulinical fusion proteins. pAlterBot was obtained from J. Middlebrook and R. Lemley at the U.S. Department of Defense. pAlterBot contains a synthetic *C. botulinum* C fragment inserted in to the pALTER-1® vector (Promega). This synthetic C fragment gene encodes the same amino acids as does the naturally occurring C fragment gene. The naturally occurring C fragment sequences, like most clostridial genes, are extremely A/T rich (Thompson *et al., supra*). This high A/T content creates expression difficulties in *E. coli* and yeast due to altered codon usage frequency and fortuitous polyadenylation sites, respectively. In order to improve the expression of C fragment proteins in *E. coli*, a synthetic version of the gene was created in which the non-preferred codons were replaced with preferred codons.

The nucleotide sequence of the *C. botulinum* C fragment gene sequences contained within pAlterBot is listed in SEQ ID NO:22. The first six nucleotides (ATGGCT) encode a methionine and alanine residue, respectively. These two amino acids result from the insertion of the *C. botulinum* C fragment sequences into the pALTER® vector and provide the initiator methionine residue. The amino acid sequence of the *C. botulinum* C fragment encoded by the sequences contained within pAlterBot is listed in SEQ ID NO:23. The first two amino acids (Met Ala) are encoded by vector-derived sequences. From the third amino acid residue onward (Arg), the amino acid sequence is identical to that found in the *C. botulinum* type A toxin gene.

The pMA1870-2680, pPA1100-2680 and pAlterBot constructs were used as progenitor plasmids to make expression constructs in which fragments of the *C. difficile* toxin A repeat domain were expressed as genetic fusions with the *C. botulinum* C fragment gene using the pMAL-c expression vector (New England Biolabs). The pMAL-c expression vector generates fusion proteins which contain the MBP at the amino-terminal end of the protein. A construct, pMBot, in which the *C. botulinum* C fragment gene was expressed as a fusion with only the MBP was constructed (Figure 25). Fusion protein expression was induced from *E. coli* strains harboring the above plasmids, and induced protein was affinity purified on an amylose resin column.

### i) Construction Of pBlueBot

In order to facilitate the cloning of the *C. botulinum* C fragment gene sequences into a number of desired constructs, the botulinal gene sequences were removed from pAlterBot and were inserted into the pBluescript plasmid (Stratagene) to generate pBlueBot (Figure 25).

pBlueBot was constructed as follows. Bacteria containing the pAlterBot plasmid were grown in medium containing tetracycline and plasmid DNA was isolated using the QIAprep-spin Plasmid Kit (Qiagen). One microgram of pAlterBot DNA was digested with *NcoI* and the resulting 3' recessed sticky end was made blunt using the Klenow fragment of DNA polymerase I (here after the Klenow fragment). The pAlterBot DNA was then digested with *HindIII* to release the botulinal gene sequences (the Bot insert) as a blunt (filled *NcoI* site)-*HindIII* fragment. pBluescript vector DNA was prepared by digesting 200 ng of pBluescript DNA with *SmaI* and *HindIII*. The digestion products from both plasmids were resolved on an agarose gel. The appropriate fragments were removed from the gel, mixed and purified utilizing the Prep-a-Gene kit (BioRad). The eluted DNA was then ligated using T4 DNA ligase and used to transform competent DH5 $\alpha$  cells (Gibco-BRL). Host cells were made competent for transformation using the calcium chloride protocol of Sambrook *et al.*, *supra* at 1.82-1.83. Recombinant clones were isolated and confirmed by restriction digestion using standard recombinant molecular biology techniques (Sambrook *et al.*, *supra*). The resultant clone, pBlueBot, contains several useful unique restriction sites flanking the Bot insert (*i.e.*, the *C. botulinum* C fragment sequences derived from pAlterBot) as shown in Figure 25.

### ii) Construction Of *C. difficile* / *C. botulinum* / MBP Fusion Proteins

Constructs encoding fusions between the *C. difficile* toxin A gene and the *C. botulinum* C fragment gene and the MBP were made utilizing the same recombinant DNA methodology outlined above; these fusion proteins contained varying amounts of the *C. difficile* toxin A repeat domain.

The pMABot clone contains a 2.4 kb insert derived from the *C. difficile* toxin A gene fused to the Bot insert (*i.e.*, the *C. botulinum* C fragment sequences derived from pAlterBot). pMABot (Figure 25) was constructed by mixing gel-purified DNA from *NotI/HindIII* digested pBlueBot (the 1.2 kb Bot fragment), *SpeI/NotI* digested pPA1100-2680 (the 2.4 kb *C. difficile* toxin A repeat fragment) and *XbaI/HindIII* digested pMAL-c vector. Recombinant clones were isolated, confirmed by restriction digestion and purified using the QIAprep-spin Plasmid

Kit (Qiagen). This clone expresses the toxin A repeats and the botulinal C fragment protein sequences as an in-frame fusion with the MBP.

The pMCABot construct contains a 1.0 kb insert derived from the *C. difficile* toxin A gene fused to the Bot insert (i.e. the *C. botulinum* C fragment sequences derived from pAlterBot). pMCABot was constructed by digesting the pMABot clone with *EcoRI* to remove the 5' end of the *C. difficile* toxin A repeat (see Figure 25, the pMAL-c vector contains a *EcoRI* site 5' to the *C. difficile* insert in the pMABot clone). The restriction sites were filled and religated together after gel purification. The resultant clone (pMCABot, Figure 25) generated an in-frame fusion between the MBP and the remaining 3' portion of the *C. difficile* toxin A repeat domain fused to the Bot gene.

The pMNABot clone contains the 1 kb *SpeI/EcoRI* (filled) fragment from the *C. difficile* toxin A repeat domain (derived from clone pPA1100-2680) and the 1.2 kb *C. botulinum* C fragment gene as a *NcoI* (filled)/*HindIII* fragment (derived from pAlterBot). These two fragments were inserted into the pMAL-c vector digested with *XbaI/HindIII*. The two insert fragments were generated by digestion of the appropriate plasmid with *EcoRI* (pPA1100-2680) or *NcoI* (pAlterBot) followed by treatment with the Klenow fragment. After treatment with the Klenow fragment, the plasmids were digested with the second enzyme (either *SpeI* or *HindIII*). All three fragments were gel purified, mixed and Prep-a-Gene purified prior to ligation. Following ligation and transformation, putative recombinants were analyzed by restriction analysis: the *EcoRI* site was found to be regenerated at the fusion junction, as was predicted for a fusion between the filled *EcoRI* and *NcoI* sites.

A construct encoding a fusion protein between the botulinal C fragment gene and the MBP gene was constructed (i.e., this fusion lacks any *C. difficile* toxin A gene sequences) and termed pMBot. The pMBot construct was made by removal of the *C. difficile* toxin A sequences from the pMABot construct and fusing the C fragment gene sequences to the MBP. This was accomplished by digestion of pMABot DNA with *StuI* (located in the pMALc polylinker 5' to the *XbaI* site) and *XbaI* (located 3' to the *NotI* site at the toxA-Bot fusion junction), filling in the *XbaI* site using the Klenow fragment, gel purifying the desired restriction fragment, and ligating the blunt ends to circularize the plasmid. Following ligation and transformation, putative recombinants were analyzed by restriction mapping of the Bot insert (i.e. the *C. botulinum* C fragment sequences).

**b) Expression Of *C. botulinum* C Fragment Fusion Proteins In  
*E. coli***

Large scale (1 liter) cultures of the pMAL-c vector, and each recombinant construct described above in (a) were grown, induced, and soluble protein fractions were isolated as described in Example 18. The soluble protein extracts were chromatographed on amylose  
5 affinity columns to isolate recombinant fusion protein. The purified recombinant fusion proteins were analyzed by running samples on SDS-PAGE gels followed by Coomassie staining and by Western blot analysis as described [Williams *et al.* (1994) *supra*]. In brief, extracts were prepared and chromatographed in column buffer (10 mM NaPO<sub>4</sub>, 0.5 M NaCl,  
10 10 mM β-mercaptoethanol, pH 7.2) over an amylose resin (New England Biolabs) column, and eluted with column buffer containing 10 mM maltose as described [Williams, *et al.* (1994), *supra*]. An SDS-PAGE gel containing the purified protein samples stained with Coomassie blue is shown in Figure 26.

In Figure 26, the following samples were loaded. Lanes 1-6 contain protein purified  
15 from *E. coli* containing the pMAL-c, pPA1870-2680, pMABot, pMNABot, pMCABot and pMBot plasmids, respectively. Lane 7 contains broad range molecular weight protein markers (BioRad).

The protein samples were prepared for electrophoresis by mixing 5 μl of eluted protein with 5 μl of 2X SDS-PAGE sample buffer (0.125 mM Tris-HCl, pH 6.8, 2 mM EDTA, 6%  
20 SDS, 20% glycerol, 0.025% bromophenol blue; β-mercaptoethanol is added to 5% before use). The samples were heated to 95°C for 5 min, then cooled and loaded on a 7.5% agarose SDS-PAGE gel. Broad range molecular weight protein markers were also loaded to allow estimation of the MW of identified fusion proteins. After electrophoresis, protein was detected generally by staining the gel with Coomassie blue.

25 In all cases the yields were in excess of 20 mg fusion protein per liter culture (see Table 36) and, with the exception of the pMCABot protein, a high percentage (*i.e.*, greater than 20-50% of total eluted protein) of the eluted fusion protein was of a MW predicted for the full length fusion protein (Figure 26). It was estimated (by visual inspection) that less than 10% of the pMCABot fusion protein was expressed as the full length fusion protein.



TABLE 36

Yield Of Affinity Purified *C. botulinum* C Fragment / MBP Fusion Proteins

Construct	Yield (mg/liter of Culture)	Percentage Of Total Soluble Protein
pMABot	24	5.0
pMCABot	34	5.0
pMNABot	40	5.5
pMBot	22	5.0
pMA1870-2680	40	4.8

These results demonstrate that high level expression of intact *C. botulinum* C fragment/*C. difficile* toxin A fusion proteins in *E. coli* is feasible using the pMAL-c expression system. These results are in contrast to those reported by H. F. LaPenotiere, *et al.* (1993), *supra*. In addition, these results show that it is not necessary to fuse the botulinal C fragment gene to the *C. difficile* toxin A gene in order to produce a soluble fusion protein using the pMAL-c system in *E. coli*.

In order to determine whether the above-described botulinal fusion proteins were recognized by anti-*C. botulinum* toxin A antibodies, Western blots were performed. Samples containing affinity-purified proteins from *E. coli* containing the pMABot, pMCABot, pMNABot, pMBot, pMA1870-2680 or pMALc plasmids were analyzed. SDS-PAGE gels (7.5% acrylamide) were loaded with protein samples purified from each expression construct. After electrophoresis, the gels were blotted and protein transfer was confirmed by Ponceau S staining (as described in Example 12b).

Following protein transfer, the blots were blocked by incubation for 1 hr at 20°C in blocking buffer [PBST (PBS containing 0.1% Tween 20 and 5% dry milk)]. The blots were then incubated in 10 ml of a solution containing the primary antibody: this solution comprised a 1/500 dilution of an anti-*C. botulinum* toxin A IgY PEG prep (described in Example 3) in blocking buffer. The blots were incubated for 1 hr at room temperature in the presence of the primary antibody. The blots were washed and developed using a rabbit anti-chicken alkaline phosphatase conjugate (Boehringer Mannheim) as the secondary antibody as follows. The rabbit anti-chicken antibody was diluted to 1 µg/ml in blocking buffer (10 ml final volume per blot) and the blots were incubated at room temperature for 1 hour in the presence of the secondary antibody. The blots were then washed successively with PBST, BBS-Tween and 50 mM Na<sub>2</sub>CO<sub>3</sub>, pH 9.5. The blots were then developed in freshly-prepared alkaline

phosphatase substrate buffer (100 µg/ml nitro blue tetrazolium, 50 µg/ml 5-bromo-chloro-indolylphosphate, 5 mM MgCl<sub>2</sub> in 50 mM Na<sub>2</sub>CO<sub>3</sub>, pH 9.5). Development was stopped by flooding the blots with distilled water and the blots were air dried.

This Western blot analysis detected anti-*C. botulinum* toxin reactive proteins in the pMABot, pMCABot, pMNABot and pMBot protein samples (corresponding to the predicted full length proteins identified above by Coomassie staining in Figure 26), but not in the pMA1100-2680 or pMALc protein samples.

These results demonstrate that the relevant fusion proteins purified on an amylose resin as described above in section a) contained immunoreactive *C. botulinum* C' fragment protein as predicted.

### EXAMPLE 23

#### Generation Of Neutralizing Antibodies

#### By Nasal Administration Of pMBot Protein

The ability of the recombinant botulinal toxin proteins produced in Example 22 to stimulate a systemic immune response against botulinal toxin epitopes was assessed. This example involved: a) the evaluation of the induction of serum IgG titers produced by nasal or oral administration of botulinal toxin-containing *C. difficile* toxin A fusion proteins and b) the *in vivo* neutralization of *C. botulinum* type A neurotoxin by anti- recombinant *C. botulinum* C' fragment antibodies.

#### a) Evaluation Of The Induction Of Serum IgG Titers Produced By Nasal Or Oral Administration Of Botulinal Toxin-Containing *C. difficile* Toxin A Fusion Proteins

Six groups containing five 6 week old CF female rats (Charles River) per group were immunized nasally or orally with one of the following three combinations using protein prepared in Example 22: (1) 250 µg pMBot protein per rat (nasal and oral); 2) 250 µg pMABot protein per rat (nasal and oral); 3) 125 µg pMBot admixed with 125 µg pMA1870-2680 per rat (nasal and oral). A second set of 5 groups containing 3 CF female rats/group were immunized nasally or orally with one of the following combinations (4) 250 µg pMNABot protein per rat (nasal and oral) or 5) 250 µg pMAL-c protein per rat (nasal and oral).

The fusion proteins were prepared for immunization as follows. The proteins (in column buffer containing 10 mM maltose) were diluted in 0.1 M carbonate buffer, pH 9.5 and administered orally or nasally in a 200  $\mu$ l volume. The rats were lightly sedated with ether prior to administration. The oral dosing was accomplished using a 20 gauge feeding needle. The nasal dosing was performed using a P-200 micro-pipettor (Gilson). The rats were boosted 14 days after the primary immunization using the techniques described above and were bled 7 days later. Rats from each group were lightly etherized and bled from the tail. The blood was allowed to clot at 37°C for 1 hr and the serum was collected.

The serum from individual rats was analyzed using an ELISA to determine the anti-*C. botulinum* type A toxin IgG serum titer. The ELISA protocol used is a modification of that described in Example 13c. Briefly, 96-well microtiter plates (Falcon, Pro-Bind Assay Plates) were coated with *C. botulinum* type A toxoid (prepared as described in Example 3a) by placing 100  $\mu$ l volumes of *C. botulinum* type A toxoid at 2.5  $\mu$ g/ml in PBS containing 0.005% thimerosal in each well and incubating overnight at 4°C. The next morning, the coating suspensions were decanted and all wells were washed three times using PBS.

In order to block non-specific binding sites, 100  $\mu$ l of blocking solution [0.5% BSA in PBS] was then added to each well and the plates were incubated for 1 hr at 37°C. The blocking solution was decanted and duplicate samples of 150  $\mu$ l of diluted rat serum added to the first well of a dilution series. The initial testing serum dilution was 1:30 in blocking solution containing 0.5% Tween 20 followed by 5-fold dilutions into this solution. This was accomplished by serially transferring 30  $\mu$ l aliquots to 120  $\mu$ l blocking solution containing 0.5% Tween 20, mixing, and repeating the dilution into a fresh well. After the final dilution, 30  $\mu$ l was removed from the well such that all wells contained 120  $\mu$ l final volume. A total of 3 such dilutions were performed (4 wells total). The plates were incubated 1 hr at 37°C. Following this incubation, the serially diluted samples were decanted and the wells were washed six times using PBS containing 0.5% Tween 20 (PBST). To each well, 100  $\mu$ l of a rabbit anti-Rat IgG alkaline phosphatase (Sigma) diluted (1/1000) in blocking buffer containing 0.5% Tween 20 was added and the plate was incubated for 1 hr at 37°C. The conjugate solutions were decanted and the plates were washed as described above, substituting 50 mM  $\text{Na}_2\text{CO}_3$ , pH 9.5 for the PBST in the final wash. The plates were developed by the addition of 100  $\mu$ l of a solution containing 1 mg/ml para-nitro phenyl phosphate (Sigma) dissolved in 50 mM  $\text{Na}_2\text{CO}_3$ , 10 mM  $\text{MgCl}_2$ , pH 9.5 to each well, and incubating the plates at room temperature in the dark for 5-45 min. The absorbency of each well was measured at

410 nm using a Dynatech MR 700 plate reader. The results are summarized in Tables 37 and 38 and represent mean serum reactivities of individual mice.

TABLE 37

Determination Of Anti-*C. botulinum* Type A Toxin Serum IgG Titers Following Immunization With *C. botulinum* C Fragment-Containing Fusion Proteins

Route of Immunization		Nasal			Oral		
Immunogen	PRE-IMMUNE	pMBot	pMBot & pMA1870-2680	pMABot	pMBot	pMBot& pMA1870-2680	pMABot
Dilution							
1:30	0.080	1.040	1.030	0.060	0.190	0.080	0.120
1:150	0.017	0.580	0.540	0.022	0.070	0.020	0.027
1:750	0.009	0.280	0.260	0.010	0.020	0.010	0.014
1:3750	0.007	0.084	0.090	0.009	0.009	0.010	0.007
# Rats Tested		5	5	5	5	2	2

Numbers represent the average values obtained from two ELISA plates, standardized utilizing the preimmune control

TABLE 38

Determination Of Anti-*C. botulinum* Type A Toxin Serum IgG Titers Following Immunization With *C. botulinum* C Fragment-Containing Fusion Proteins

Route of Immunization		Nasal		Oral	
Immunogen	PRE-IMMUNE	pMBot	pMABot	pMNABot	pMNABot
Dilution					
1:30	0.040	0.557	0.010	0.015	0.010
1:150	0.009	0.383	0.001	0.003	0.002
1:750	0.001	0.140	0.000	0.000	0.000
1:3750	0.000	0.040	0.000	0.000	0.000
# Rats Tested		1	1	3	3

The above ELISA results demonstrate that reactivity against the botulinal fusion proteins was strongest when the route of administration was nasal; only weak responses were stimulated when the botulinal fusion proteins were given orally. Nasally delivered pMBot and pMBot admixed with pMA1870-2680 invoked the greatest serum IgG response. These results show that only the pMBot protein is necessary to induce this response, since the addition of the pMA1870-2680 protein did not enhance antibody response (Table 37). Placement of the *C. difficile* toxin A fragment between the MBP and the *C. botulinum* C fragment protein

dramatically reduced anti-bot IgG titer (see results using pMABot, pMCABot and pMNABot proteins).

This study demonstrates that the pMBot protein induces a strong serum IgG response directed against *C. botulinum* type A toxin when nasally administered.

5  
b) ***In Vivo* Neutralization Of *C. botulinum* Type A Neurotoxin  
By Anti- Recombinant *C. botulinum* C Fragment Antibodies**

The ability of the anti-*C. botulinum* type A toxin antibodies generated by nasal administration of recombinant botulinal fusion proteins in rats (Example 22) to neutralize *C. botulinum* type A toxin was tested in a mouse neutralization model. The mouse model is the art accepted method for detection of botulinal toxins in body fluids and for the evaluation of anti-botulinal antibodies [E.J. Schantz and D.A. Kautter, J. Assoc. Off. Anal. Chem. 61:96 (1990) and Investigational New Drug (BB-IND-3703) application by the Surgeon General of the Department of the Army to the Federal Food and Drug Administration]. The anti-*C. botulinum* type A toxin antibodies were prepared as follows.

15 Rats from the group given pMBot protein by nasal administration were boosted a second time with 250 µg pMBot protein per rat and serum was collected 7 days later. Serum from one rat from this group and from a preimmune rat was tested for anti-*C. botulinum* type A toxin neutralizing activity in the mouse neutralization model described below.

20 The LD<sub>50</sub> of a solution of purified *C. botulinum* type A toxin complex, obtained from Dr. Eric Johnson (University of Wisconsin Madison), was determined using the intraperitoneal (IP) method of Schantz and Kautter [J. Assoc. Off. Anal. Chem. 61:96 (1978)] using 18-22 gram female ICR mice and was found to be 3500 LD<sub>50</sub>/ml. The determination of the LD<sub>50</sub> was performed as follows. A Type A toxin standard was prepared by dissolving purified type A toxin complex in 25 mM sodium phosphate buffer, pH 6.8 to yield a stock toxin solution of 3.15 x 10<sup>7</sup> LD<sub>50</sub>/mg. The OD<sub>278</sub> of the solution was determined and the concentration was adjusted to 10-20 µg/ml. The toxin solution was then diluted 1:100 in gel-phosphate (30 mM phosphate, pH 6.4; 0.2% gelatin). Further dilutions of the toxin solution were made as shown below in Table 39. Two mice were injected IP with 0.5 ml of each dilution shown and the mice were observed for symptoms of botulism for a period of 72 hours.

25  
30

TABLE 39

Determination Of The LD<sub>50</sub> Of Purified *C. botulinum* Type A Toxin Complex

Dilution	Number Dead At 72 hr
1:320	2/2
1:640	2/2
1:1280	2/2
1:2560	0/2 (sick after 72 hr)
1:5120	0/2 (no symptoms)

From the results shown in Table 39, the toxin titer was assumed to be between 2560 LD<sub>50</sub>/ml and 5120 LD<sub>50</sub>/ml (or about 3840 LD<sub>50</sub>/ml). This value was rounded to 3500 LD<sub>50</sub>/ml for the sake of calculation.

The amount of neutralizing antibodies present in the serum of rats immunized nasally with pMBot protein was then determined. Serum from two rats boosted with pMBot protein as described above and preimmune serum from one rat was tested as follows. The toxin standard was diluted 1:100 in gel-phosphate to a final concentration of 350 LD<sub>50</sub>/ml. One milliliter of the diluted toxin standard was mixed with 25  $\mu$ l of serum from each of the three rats and 0.2 ml of gel-phosphate. The mixtures were incubated at room temperature for 30 min with occasional mixing. Each of two mice were injected with IP with 0.5 ml of the mixtures. The mice were observed for signs of botulism for 72 hr. Mice receiving serum from rats immunized with pMBot protein neutralized this challenge dose. Mice receiving preimmune rat serum died in less than 24 hr.

The amount of neutralizing anti-toxin antibodies present in the serum of rats immunized with pMBot protein was then quantitated. Serum antibody titrations were performed by mixing 0.1 ml of each of the antibody dilutions (see Table 40) with 0.1 ml of a 1:10 dilution of stock toxin solution ( $3.5 \times 10^4$  LD<sub>50</sub>/ml) with 1.0 ml of gel-phosphate and injecting 0.5 ml IP into 2 mice per dilution. The mice were then observed for signs of botulism for 3 days (72 hr). The results are tabulated in Table 39.

As shown in Table 40 pMBot serum neutralized *C. botulinum* type A toxin complex when used at a dilution of 1:320 or less. A mean neutralizing value of 168 IU/ml was obtained for the pMBot serum (an IU is defined as 10,000 mouse LD<sub>50</sub>). This value translates to a circulating serum titer of about 3.7 IU/mg of serum protein. This neutralizing titer is comparable to the commercially available bottled concentrated (Connaught Laboratories, Ltd.) horse anti-*C. botulinum* antiserum. A 10 ml vial of Connaught antiserum contains about 200

mg/ml of protein:each ml can neutralize 750 IU of *C. botulinum* type A toxin. After administration of one vial to a human, the circulating serum titer of the Connaught preparation would be approximately 25 IU/ml assuming an average serum volume of 3 liters). Thus, the circulating anti-*C. botulinum* titer seen in rats nasally immunized with pMBot protein (168 IU/ml) is 6.7 time higher than the necessary circulation titer of anti-*C. botulinum* antibody needed to be protective in humans.

TABLE 40

Quantitation Of Neutralizing Antibodies In pMBot Sera

Dilution	pMBot <sup>a</sup>	
	Rat 1	Rat 2
1:20	2/2	2/2
1:40	2/2	2/2
1:80	2/2	2/2
1:160	2/2	2/2
1:320	2/2 <sup>b</sup>	2/2 <sup>b</sup>
1:640	0/2	0/2
1:1280	0/2	0/2
1:2560	0/2	0/2

<sup>a</sup> Numbers represent the number of mice surviving at 72 hours which received serum taken from rats immunized with the pMBot protein.

<sup>b</sup> These mice survived but were sick after 72 hr.

These results demonstrate that antibodies capable of neutralizing *C. botulinum* type A toxin are induced when recombinant *C. botulinum* C fragment fusion protein produced in *E. coli* is used as an immunogen.

## EXAMPLE 24

Production Of Soluble *C. botulinum* C Fragment  
Protein Substantially Free Of Endotoxin Contamination

Example 23 demonstrated that neutralizing antibodies are generated by immunization with the pMBot protein expressed in *E. coli*. These results showed that the pMBot fusion protein is a good vaccine candidate. However, immunogens suitable for use as vaccines should be pyrogen-free in addition to having the capability of inducing neutralizing

antibodies. Expression clones and conditions that facilitate the production of *C. botulinum* C fragment protein for utilization as a vaccine were developed.

The example involved: (a) determination of pyrogen content of the pMBot protein; (b) generation of *C. botulinum* C fragment protein free of the MBP; (c) expression of *C. botulinum* C fragment protein using various expression vectors; and (d) purification of soluble *C. botulinum* C fragment protein substantially free of significant endotoxin contamination.

**a) Determination Of The Pyrogen Content Of The pMBot Protein**

In order to use a recombinant antigen as a vaccine in humans or other animals, the antigen preparation must be shown to be free of pyrogens. The most significant pyrogen present in preparations of recombinant proteins produced in gram-negative bacteria, such as *E. coli*, is endotoxin [F.C. Pearson, *Pyrogens: endotoxins, LAL testing and depyrogenation*, (1985) Marcel Dekker, New York, pp. 23-56]. To evaluate the utility of the pMBot protein as a vaccine candidate, the endotoxin content in MBP fusion proteins was determined.

The endotoxin content of recombinant protein samples was assayed utilizing the Limulus assay (LAL kit: Associates of Cape Cod ) according to the manufacturer's instructions. Samples of affinity-purified pMal-c protein and pMA1870-2680 were found to contain high levels of endotoxin [ $>50,000$  EU/mg protein; EU (endotoxin unit)]. This suggested that MBP- or toxin A repeat-containing fusions with the botulin C fragment should also contain high levels of endotoxin. Accordingly, removal of endotoxin from affinity-purified pMal-c and pMBot protein preparations was attempted as follows.

Samples of pMal-c and pMBot protein were depyrogenated with polymyxin to determine if the endotoxin could be easily removed. The following amount of protein was treated: 29 ml at  $4.8 \text{ OD}_{280}/\text{ml}$  for pMal-c and 19 mls at  $1.44 \text{ OD}_{280}/\text{ml}$  for pMBot. The protein samples were dialyzed extensively against PBS and mixed in a 50 ml tube (Falcon) with 0.5 ml PBS-equilibrated polymyxin B (Affi-Prep Polymyxin, BioRad). The samples were allowed to mix by rotating the tubes overnight at  $4^\circ\text{C}$ . The polymyxin was pelleted by centrifugation for 30 min in a bench top centrifuge at maximum speed (approximately  $2000 \times g$ ) and the supernatant was removed. The recovered protein (in the supernatant) was quantified by  $\text{OD}_{280}$ , and the endotoxin activity was assayed by LAL. In both cases only approximately 1/3 of the input protein was recovered and the polymyxin-treated protein retained significant endotoxin contamination (approximately 7000 EU/mg of pMBot).



The depyrogenation experiment was repeated using an independently purified pMal-c protein preparation and similar results were obtained. From these studies it was concluded that significant levels of endotoxin copurifies with these MBP fusion proteins using the amylose resin. Furthermore, this endotoxin cannot be easily removed by polymyxin treatment.

These results suggest that the presence of the MBP sequences on the fusion protein complicated the removal of endotoxin from preparations of the pMBot protein.

**b) Generation Of *C. botulinum* C Fragment Protein Free Of The MBP**

It was demonstrated that the pMBot fusion protein could not be easily purified from contaminating endotoxin in section a) above. The ability to produce a pyrogen-free (*e.g.*, endotoxin-free) preparation of soluble botulinum C fragment protein free of the MBP tag was next investigated. The pMBot expression construct was designed to facilitate purification of the botulinum C fragment from the MBP tag by cleavage of the fusion protein by utilizing an engineered Factor Xa cleavage site present between the MBP and the botulinum C fragment. The Factor Xa cleavage was performed as follows.

Factor Xa (New England Biolabs) was added to the pMBot protein (using a 0.1-1.0% Factor Xa/pMBot protein ratio) in a variety of buffer conditions [*e.g.*, PBS-NaCl (PBS containing 0.5 M NaCl), PBS-NaCl containing 0.2% Tween 20, PBS, PBS containing 0.2% Tween 20, PBS-C (PBS containing 2 mM CaCl<sub>2</sub>), PBS-C containing either 0.1 or 0.5 % Tween 20, PBS-C containing either 0.1 or 0.5% NP-40, PBS-C containing either 0.1 or 0.5% Triton X-100, PBS-C containing 0.1% sodium deoxycholate, PBS-C containing 0.1% SDS]. The Factor Xa digestions were incubated for 12-72 hrs at room temperature.

The extent of cleavage was assessed by Western blot or Coomassie blue staining of proteins following electrophoresis on denaturing SDS-PAGE gels, as described in Example 22. Cleavage reactions (and control samples of uncleaved pMBot protein) were centrifuged for 2 min in a microfuge to remove insoluble protein prior to loading the samples on the gel. The Factor Xa treated samples were compared with uncleaved, uncentrifuged pMBot samples on the same gel. The results of this analysis is summarized below.

1) Most (about 90%) pMBot protein could be removed by centrifugation, even when uncleaved control samples were utilized. This indicated that the pMBot fusion protein was not fully soluble (*i.e.*, it exists as a suspension rather than as a solution). [This result was

consistent with the observation that most affinity-purified pMBot protein precipitates after long term storage (>2 weeks) at 4°C. Additionally, the majority (*i.e.*, 75%) of induced pMBot protein remains in the pellet after sonication and clarification of the induced *E. coli*. Resuspension of these insoluble pellets in PBS followed by sonication results in partial solubilization of the insoluble pMBot protein in the pellets.)

2) The portion of pMBot protein that is fully in solution (about 10% of pMBot protein) is completely cleaved by Factor Xa, but the cleaved (released) botulinal C fragment is relatively insoluble such that only the cleaved MBP remains fully in solution.

3) None of the above reaction conditions enhanced solubility without also reducing effective cleavage. Conditions that effectively solubilized the cleaved botulinal C fragment were not identified.

4) The use of 0.1% SDS in the buffer used for Factor Xa cleavage enhanced the solubility of the pMBot protein (all of pMBot protein was soluble). However, the presence of the SDS prevented any cleavage of the fusion protein with Factor Xa.

5) Analysis of pelleted protein from the cleavage reactions indicated that both full length pMBot (*i.e.*, uncleaved) and cleaved botulinal C fragment protein precipitated during incubation.

These results demonstrate that purification of soluble botulinal C fragment protein after cleavage of the pMBot fusion protein is complicated by the insolubility of both the pMBot protein and the cleaved botulinal C fragment protein.

#### c) Expression Of *C. botulinum* C Fragment Using Various Expression Vectors

In order to determine if the solubility of the botulinal C fragment was enhanced by expressing the C fragment protein as a native protein, an N-terminal His-tagged protein or as a fusion with glutathione-S-transferase (GST), alternative expression plasmids were constructed. These expression constructs were generated utilizing the methodologies described in Example 22. Figure 27 provides a schematic representation of the vectors described below.

In Figure 27, the following abbreviations are used. pP refers to the pET23 vector. pHIS refers to the pETHisa vector. pBlue refers to the pBluescript vector. pM refers to the pMAL-c vector and pG refers to the pGEX5T vector (described in Example 11). The solid black lines represent *C. botulinum* C fragment gene sequences; the solid black ovals represent the MBP; the hatched ovals represent GST; "HHHHH" represents the poly-histidine tag. In

Figure 27. when the name for a restriction enzyme appears inside parenthesis, this indicates that the restriction site was destroyed during construction. An asterisk appearing with the name for a restriction enzyme indicates that this restriction site was recreated at a cloning junction.

#### i) Construction Of pPBot

In order to express the *C. botulinum* C fragment as a native (*i.e.*, non-fused) protein, the pPBot plasmid (shown schematically in Figure 27) was constructed as follows. The C fragment sequences present in pAlterBot (Example 22) were removed by digestion of pAlterBot with *Nco*I and *Hind*III. The *Nco*I/*Hind*III C fragment insert was ligated to pETHisa vector (described in Example 18b) which was digested with *Nco*I and *Hind*III. This ligation creates an expression construct in which the *Nco*I-encoded methionine of the botulinal C fragment is the initiator codon and directs expression of the native botulinal C fragment. The ligation products were used to transform competent BL21(DE3)pLysS cells (Novagen). Recombinant clones were identified by restriction mapping.

#### ii) Construction Of pHisBot

In order to express the *C. botulinum* C fragment containing a poly-histidine tag at the amino-terminus of the recombinant protein, the pHisBot plasmid (shown schematically in Figure 27) was constructed as follows. The *Nco*I/*Hind*III botulinal C fragment insert from pAlterbot was ligated into the pETHisa vector which was digested with *Nhe*I and *Hind*III. The *Nco*I (on the C fragment insert) and *Nhe*I (on the pETHisa vector) sites were filled in using the Klenow fragment prior to ligation; these sites were then blunt end ligated (the *Nde*I site was regenerated at the clone junction as predicted). The ligation products were used to transform competent BL21(DE3)pLysS cells and recombinant clones were identified by restriction mapping.

The resulting pHisBot clone expresses the botulinal C fragment protein with a histidine-tagged N-terminal extension having the following sequence: MetGlyHisHisHisHisHisHisHisHisHisHisSerSerGlyHisIleGluGlyArgHisMetAla<sub>2</sub> (SEQ ID NO:24); the amino acids encoded by the botulinal C fragment gene are underlined and the vector encoded amino acids are presented in plain type. The nucleotide sequence present in the pETHisa vector which encodes the pHisBot fusion protein is listed in SEQ ID NO:25. The amino acid sequence of the pHisBot protein is listed in SEQ ID NO:26.

### iii) Construction Of pGBot

The botulinal C fragment protein was expressed as a fusion with the glutathione-S-transferase protein by constructing the pGBot plasmid (shown schematically in Figure 27). This expression construct was created by cloning the *NcoI/SalI* C fragment insert present in pBlueBot (Example 22) into the pGEX3T vector which was digested with *SmaI* and *XhoI*.  
5 The *NcoI* site (present on the botulinal fragment) was made blunt prior to ligation using the Klenow fragment. The ligation products were used to transform competent BL21 cells.

Each of the above expression constructs were tested by restriction digestion to confirm the integrity of the constructs.

10 Large scale (1 liter) cultures of pPBot [BL21(DE3)pLysS host], pHisBot [BL21(DE3)pLysS host] and pGBot (BL21 host) were grown in 2X YT medium and induced (using IPTG to 0.8-1.0 mM) for 3 hrs as described in Example 22. Total, soluble and insoluble protein preparations were prepared from 1 ml aliquots of each large scale culture [Williams *et al.* (1994), *supra*] and analyzed by SDS-PAGE. No obvious induced band was  
15 detectable in the pPBot or pHisBot samples by Coomassie staining, while a prominent insoluble band of the anticipated MW was detected in the pGBot sample. Soluble lysates of the pGBot large scale (resuspended in PBS) or pHisBot large scale [resuspended in Novagen 1X binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9)] cultures were prepared and used to affinity purify soluble affinity-tagged protein as follows.

20 The pGBot lysate was affinity purified on a glutathione-agarose resin (Pharmacia) exactly as described in Smith and Corcoran [Current Protocols in Molecular Biology, Supplement 28 (1994), pp. 16.7.1-16.7.7]. The pHisBot protein was purified on the His-Bind resin (Novagen) utilizing the His-bind buffer kit (Novagen) exactly as described by manufacturer.

25 Samples from the purification of both the pGBot and pHisBot proteins (including uninduced, induced, total, soluble, and affinity-purified eluted protein) were resolved on SDS-PAGE gels. Following electrophoresis, proteins were analyzed by Coomassie staining or by Western blot detection utilizing a chicken anti-*C. botulinum* Type A toxoid antibody (as described in Example 22).

30 These studies showed that the pGBot protein was almost entirely insoluble under the utilized conditions, while the pHisBot protein was soluble. Affinity purification of the pHisBot protein on this first attempt was inefficient, both in terms of yield (most of the

immunoreactive botulinal protein did not bind to the His-bind resin) and purity (the botulinal protein was estimated to comprise approximately 20% of the total eluted protein).

**d) Purification Of Soluble *C. botulinum* C Fragment Protein  
Substantially Free Of Endotoxin Contamination**

The above studies showed that the pHisBot protein was expressed in *E. coli* as a soluble protein. However, the affinity purification of this protein on the His-bind resin was very inefficient. In order to improve the affinity purification of the soluble pHisBot protein (in terms of both yield and purity), an alternative poly-histidine binding affinity resin (Ni-NTA resin; Qiagen) was utilized. The Ni-NTA resin was reported to have a superior binding affinity ( $K_d = 1 \times 10^{-13}$  at pH 8.0; Qiagen user manual) relative to the His-bind resin.

A soluble lysate (in Novagen 1X binding buffer) from an induced 1 liter 2X YT culture was prepared as described above. Briefly, the culture of pHisBot [BI21(DE3)pLysS host] was grown at 37°C to an  $OD_{600}$  of 0.7 in 1 liter of 2X YT medium containing 100 µg/ml ampicillin, 34 µg/ml chloramphenicol and 0.2% glucose. Protein expression was induced by the addition of IPTG to 1 mM. Three hours after the addition of the IPTG, the cells were cooled for 15 min in a ice water bath and then centrifuged 10 min at 5000 rpm in a JA10 rotor (Beckman) at 4°C. The pellets were resuspended in a total volume of 40 mls Novagen 1X binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9), transferred to two 35 ml Oakridge tubes and frozen at -70°C for at least 1 hr. The tubes were thawed and the cells were lysed by sonication (4 X 20 second bursts using a Branson Sonifier 450 with a power setting of 6-7) on ice. The suspension was clarified by centrifugation for 20 min at 9,000 rpm (10,000 x g) in a JA-17 rotor (Beckman).

The soluble lysate was brought to 0.1% NP40 and then was batch absorbed to 7 ml of a 1:1 slurry of Ni-NTA resin:binding buffer by stirring for 1 hr at 4°C. The slurry was poured into a column having an internal diameter of 1 or 2.5 cm (BioRad). The column was then washed sequentially with 15 mls of Novagen 1X binding buffer containing 0.1% NP40, 15 ml of Novagen 1X binding buffer, 15 ml wash buffer (60 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9) and 15 ml NaHPO<sub>4</sub> wash buffer (50 mM NaHPO<sub>4</sub>, pH 7.0, 0.3 M NaCl, 10 % glycerol). The bound protein was eluted by protonation of the resin using elution buffer (50 mM NaHPO<sub>4</sub>, pH 4.0, 0.3 M NaCl, 10 % glycerol). The eluted protein was stored at 4°C.

Samples of total, soluble and eluted protein were resolved by SDS-PAGE. Protein samples were prepared for electrophoresis as described in Example 22b. Duplicate gels were stained with Coomassie blue to visualize the resolved proteins and *C. botulinum* type A toxin-reactive protein was detected by Western blot analysis as described in Example 22b. A representative Coomassie stained gel is shown in Figure 28. In Figure 28, the following samples were loaded on the 12.5% acrylamide gel. Lanes 1-4 contain respectively total protein, soluble protein, soluble protein present in the flow-through of the Ni-NTA column and affinity-purified pHisBot protein (*i.e.*, protein released from the Ni-NTA resin by protonation). Lane 5 contains high molecular weight protein markers (BioRad).

The purification of pHisBot protein resulted in a yield of 7 mg of affinity purified protein from a 1 liter starting culture of BL21(DE3)pLysS cells harboring the pHisBot plasmid. The yield of purified pHisBot protein represented approximately 0.4% of the total soluble protein in the induced culture. Analysis of the purified pHisBot protein by SDS-PAGE revealed that at least 90-95% of the protein was present as a single band (Figure 28) of the predicted MW (50 kD). This 50 kD protein band was immunoreactive with anti-*C. botulinum* type A toxin antibodies. The extinction coefficient of the protein preparation was determined to be 1.4 (using the Pierce BCA assay) or 1.45 (using the Lowry assay) (OD<sub>280</sub> per 1 mg/ml solution).

Samples of pH neutralized eluted pHisBot protein were resolved on a KB 803 HPLC column (Shodex). Although His-tagged proteins are retained by this sizing column (perhaps due to the inherent metal binding ability of the proteins), the relative mobility of the pHisBot protein was consistent with that expected for a non-aggregated protein in solution. Most of the induced pHisBot protein was determined to be soluble under the growth and solubilization conditions utilized above (*i.e.*, greater than 90% of the pHisBot protein was found to be soluble as judged by comparison of the levels of pHisBot protein seen in total and soluble protein samples prepared from BL21(DE3)pLysS cells containing the pHisBot plasmid). SDS-PAGE analysis of samples obtained after centrifugation, extended storage at -20°C, and at least 2 cycles of freezing and thawing detected no protein loss (due to precipitation), indicating that the pHisBot protein is soluble in the elution buffer (*i.e.*, 50 mM NaHPO<sub>4</sub>, pH 4.0, 0.3 M NaCl, 10 % glycerol).

Determination of endotoxin contamination in the affinity purified pHisBot preparation (after pH neutralization) using the LAL assay (Associates of Cape Cod) detected no significant endotoxin contamination. The assay was performed using the endpoint

chromogenic method (without diazo-coupling) according to the manufacturer's instructions. This method can detect concentrations of endotoxin greater than or equal to 0.03 EU/ml (EU refers to endotoxin units). The LAL assay was run using 0.5 ml of a solution comprising 0.5 mg pHisBot protein in 50 mM NaHPO<sub>4</sub>, pH 7.0, 0.3 M NaCl, 10 % glycerol: 30-60 EU were detected in the 0.5 ml sample. Therefore, the affinity purified pHisBot preparation contains 60-120 EU/mg of protein. FDA Guidelines for the administration of parenteral drugs require that a composition to be administered to a human contain less than 5 EU/kg body weight (The average human body weight is 70 kg: therefore up to 349 EU units can be delivered in a parental dose.). Because very small amount of protein are administered in a vaccine preparation (generally in the range of 10-500 µg of protein), administration of affinity purified pHisBot containing 60-120 EU/mg protein would result in delivery of only a small percentage of the permissible endotoxin load. For example, administration of 10-500 µg of purified pHisBot to a 70 kg human, where the protein preparation contains 60 EU/mg protein, results in the introduction of only 0.6 to 30 EU [*i.e.*, 0.2 to 8.6% of the maximum allowable endotoxin burden per parenteral dose (less than 5 EU/kg body weight)].

The above results demonstrate that endotoxin (LPS) does not copurify with the pHisBot protein using the above purification scheme. Preparations of recombinantly produced pHisBot protein containing lower levels of endotoxin (less than or equal to 2 EU/ mg recombinant protein) may be produced by washing the Ni-NTA column with wash buffer until the OD<sub>280</sub> returns to baseline levels (*i.e.*, until no more UV-absorbing material comes off of the column).

The above results illustrate a method for the production and purification of soluble, botulinal C' fragment protein substantially free of endotoxin.

## EXAMPLE 25

### Optimization Of The Expression And Purification Of pHisBot Protein

The results shown in Example 24d demonstrated that the pHisBot protein is an excellent candidate for use as a vaccine as it could be produced as a soluble protein in *E. coli* and could be purified free of pyrogen activity. In order to optimize the expression and purification of the pHisBot protein, a variety of growth and purification conditions were tested.

a) **Growth Parameters**

i) **Host Strains**

The influence of the host strain utilized upon the production of soluble pHisBot protein was investigated. A large scale purification of pHisBot was performed [as described in Example 24d above] using the BL21(DE3) host (Novagen) rather than the BL21(DE3)pLysS host. The deletion of the pLysS plasmid in the BL21(DE3) host yielded higher levels of expression due to de-repression of the plasmid's T7-lac promoter. However, the yield of affinity-purified soluble recombinant protein was very low (approximately 600 µg/ liter culture) when purified under conditions identical to those described in Example 24d above. This result was due to the fact that expression in the BL21(DE3) host yielded very high level expression of the pHisBot protein as insoluble inclusion bodies as shown by SDS-PAGE analysis of protein prepared from induced BL21(DE3) cultures (Figure 29, lanes 1-7, described below). These results demonstrate that the pHisBot protein is not inherently toxic to *E. coli* cells and can be expressed to high levels using the appropriate promoter/host combination.

Figure 29 shows a Coomassie blue stained SDS-PAGE gel (12.5% acrylamide) onto which extracts prepared from BL21(DE3) cells containing the pHisBot plasmid were loaded. Each lane was loaded with 2.5 µl protein sample mixed with 2.5 µl of 2X SDS sample buffer. The samples were handled as described in Example 22b. The following samples were applied to the gel. Lanes 1-7 contain protein isolated from the BL21(DE3) host. Lanes 8-14 contain proteins isolated from the BL21(DE3)pLysS host. Total protein was loaded in lanes 1, 2, 4, 6, 8, 10 and 12. Soluble protein was loaded in Lanes 3, 5, 7, 9, 11 and 13. Lane 1 contains protein from uninduced host cells. Lanes 2-13 contain protein from host cells induced for 3 hours. IPTG was added to a final concentration of 0.1 mM (Lanes 6-7), 0.3 mM (Lanes 4-5) or 1.0 mM (Lanes 2, 3, 8-13). The cultures were grown in LB broth (Lanes 8-9), 2X YT broth (Lanes 10-11) or terrific broth (Lanes 1-7, 12-13). The pHisBot protein seen in Lanes 3, 5 and 7 is insoluble protein which spilled over from Lanes 2, 4 and 6, respectively. High molecular weight protein markers (BioRad) were loaded in Lane 14.

A variety of expression conditions were tested to determine if the BL21(DE3) host could be utilized to express soluble pHisBot protein at suitably high levels (*i.e.*, about 10 mg/ml). The conditions altered were temperature (growth at 37 or 30°C), culture medium (2X YT, LB or Terrific broth) and inducer levels (0.1, 0.3 or 1.0 mM IPTG). All combinations of these variables were tested and the induction levels and solubility was then



assessed by SDS-PAGE analysis of total and soluble extracts [prepared from 1 ml samples as described in Williams *et al.*, (1994), *supra*].

All cultures were grown in 15 ml tubes (Falcon #2057). All culture medium was prewarmed overnight at the appropriate temperature and were supplemented with 100 µg/ml ampicillin and 0.2% glucose. Terrific broth contains 12 g/l bacto-tryptone, 24 g/l bacto-yeast extract and 100 ml/l of a solution comprising 0.17 M  $\text{KH}_2\text{PO}_4$ , 0.72 M  $\text{K}_2\text{HPO}_4$ . Cultures were grown in a incubator on a rotating wheel (to ensure aeration) to an  $\text{OD}_{600}$  of approximately 0.4, and induced by the addition of IPTG. In all cases, high level expression of insoluble pHisBot protein was observed, regardless of temperature, medium or inducer concentration.

The effect of varying the concentration of IPTG upon 2X YT cultures grown at 23°C was then investigated. IPTG was added to a final concentration of either 1 mM, 0.1 mM, 0.05 mM or 0.01 mM. At this temperature, similar levels of pHis Bot protein was induced in the presence of either 1 or 0.1 mM IPTG; these levels of expression was lower than that observed at higher temperatures. Induced protein levels were reduced at 0.05 mM IPTG and absent at 0.01 mM IPTG (relative to 1.0 and 0.1 mM IPTG inductions at 23°C). However, no conditions were observed in which the induced pHisBot protein was soluble in this host. Thus, although expression levels are superior in the BL21(DE3) host (as compared to the BL21(DE3)pLysS host), conditions that facilitate the production of soluble protein in this host could not be identified.

These results demonstrate that production of soluble pHisBot protein was achieved using the BL21(DE3)pLysS host in conjunction with the T7-lac promoter.

## ii) Effect Of Varying Temperature, Medium And IPTG Concentration And Length Of Induction

The effect growing the host cells in various mediums upon the expression of recombinant botulinal protein from the pHisBot expression construct [in the BL21(DE3)pLysS host] was investigated. BL21(DE3)pLysS cells containing the pHisBot plasmid were grown in either LB, 2X YT or Terrific broth at 37°C. The cells were induced using 1 mM IPTG for a 3 hr induction period. Expression of pHisBot protein was found to be the highest when the cells were grown in 2X YT broth (see Figure 29, lanes 8-13).

The cells were then grown at 30°C in 2X YT broth and the concentration of IPTG was varied from 1.0, 0.3 or 0.1 mM and the length of induction was either 3 or 5 hours.

Expression of pHisBot protein was similar at all 3 inducer concentrations utilized and the levels of induced protein were higher after a 5 hr induction as compared to a 3 hr induction.

Using the conditions found to be optimal for the expression of pHisBot protein, a large scale culture was grown in order to provide sufficient material for a large scale purification of the pHisBot protein. Three 1 liter cultures were grown in 2X YT medium containing 100  $\mu\text{g/ml}$  ampicillin, 34  $\mu\text{g/ml}$  chloramphenicol and 0.2% glucose. The cultures were grown at 30°C and were induced with 1.0 mM IPTG for a 5 hr period. The cultures were harvested and a soluble lysate were prepared as described in Example 18. A large scale purification was performed as described in Example 24d with the exception that except the soluble lysate was batch absorbed for 3 hours rather than for 1 hour. The final yield was 13 mg pHisBot protein/liter culture. The pHisBot protein represented 0.75% of the total soluble protein.

The above results demonstrate growth conditions under which soluble pHisBot protein is produced (*i.e.*, use of the BL21(DE3)pLysS host, 2X YT medium, 30°C, 1.0 mM IPTG for 5 hours).

#### **b) Optimization Of Purification Parameters**

For optimization of purification conditions, large scale cultures (3 X 1 liter) were grown at 30°C and induced with 1 mM IPTG for 5 hours as described above. The cultures were pooled, distributed to centrifuge bottles, cooled and pelleted as described in Example 24d. The cell pellets were frozen at -70°C until used. Each cell pellet represented 1/3 of a liter starting culture and individual bottles were utilized for each optimization experiment described below. This standardized the input bacteria used for each experiment, such that the yields of affinity purified pHisBot protein could be compared between different optimization experiments.

#### **i) Binding Specificity (pH Protonation)**

A lysate of pHisBot culture was prepared in PBS (pH 8.0) and applied to a 3 ml Ni-NTA column equilibrated in PBS (pH 8.0) using a flow rate of 0.2 ml/min (3-4 column volumes/hr) using an Econo chromatography system (BioRad). The column was washed with PBS (pH 8.0) until the absorbance ( $\text{OD}_{280}$ ) of the elute was at baseline levels. The flow rate was then increased to 2 ml/min and the column was equilibrated in PBS (pH 7.0). A pH gradient (pH 7.0 to 4.0 in PBS) was applied in order to elute the bound pHisBot protein from the column. Fractions were collected and aliquots were resolved on SDS-PAGE gels. The

PAGE gels were subjected to Western blotting and the pHisBot protein was detected using a chicken anti-*C. botulinum* Type A toxoid antibody as described in Example 22.

From the Western blot analysis it was determined that the pHisBot protein begins to elute from the Ni-NTA column at pH 6.0. This is consistent with the predicted elution of a His-tagged protein monomer at pH 5.9.

These results demonstrate that the pH at which the pHisBot protein is protonated (released) from Ni-NTA resin in PBS buffer is pH 6.0.

## ii) Binding Specificity (Imidazole Competition)

In order to define purification conditions under which the native *E. coli* proteins could be removed from the Ni-NTA column while leaving the pHisBot protein bound to the column, the following experiment was performed. A lysate of pHisBot culture was prepared in 50 mM NaHPO<sub>4</sub>, 0.5 M NaCl, 8 mM imidazole (pH 7.0). This lysate was applied to a 3 ml Ni-NTA column equilibrated in 50 mM NaHPO<sub>4</sub>, 0.5 M NaCl (pH 7.0) using an Econo chromatography system (BioRad). A flow rate of 0.2 ml/min (3-4 column volumes/hr) was utilized. The column was washed with 50 mM NaHPO<sub>4</sub>, 0.5 M NaCl (pH 7.0) until the absorbance of the elute returned to baseline. The flow rate was then increased to 2 ml/min.

The column was eluted using an imidazole step gradient [in 50 mM NaHPO<sub>4</sub>, 0.5 M NaCl (pH 7.0)]. Elution steps were 20 mM, 40 mM, 60 mM, 80 mM, 100 mM, 200 mM, 1.0 M imidazole, followed by a wash using 0.1 mM EDTA (to strip the nickel from the column and remove any remaining protein). In each step, the wash was continued until the OD<sub>280</sub> returned to baseline. Fractions were resolved on SDS-PAGE gels, Western blotted, and pHisBot protein detected using a chicken anti-*C. botulinum* Type A toxoid antibody as described in Example 22. Duplicate gels were stained with Coomassie blue to detect eluted protein in each fraction.

The results of the PAGE analysis showed that most of the non-specifically binding bacterial protein was removed by the 20 mM imidazole wash, with the remaining bacterial proteins being removed in the 40 and 60 mM imidazole washes. The pHisBot protein began to elute at 100 mM imidazole and was quantitatively eluted in 200 mM imidazole.

These results precisely defined the window of imidazole wash stringency that optimally removes *E. coli* proteins from the column while specifically retaining the pHisBot protein in this buffer. These results provided conditions under which the pHisBot protein can be purified free of contaminating host proteins.

### iii) Purification Buffers And Optimized Purification Protocols

A variety of purification parameters were tested during the development of an optimized protocol for batch purification of soluble pHisBot protein. The results of these analyses are summarized below.

Batch purifications were performed (as described in Example 24d) using several buffers to determine if alternative buffers could be utilized for binding of the pHisBot protein to the Ni-NTA column. It was determined that quantitative binding of pHisBot protein to the Ni-NTA resin was achieved in either Tris-HCl (pH 7.9) or NaHPO<sub>4</sub> (pH 8.0) buffers. Binding of the pHisBot protein in NaHPO<sub>4</sub> buffer was not inhibited using 5 mM, 8 mM or 60 mM imidazole. Quantitative elution of bound pHisBot protein was obtained in buffers containing 50 mM NaHPO<sub>4</sub>, 0.3 M NaCl (pH 3.5-4.0), with or without 10% glycerol. However, quantitation of soluble affinity purified pHisBot protein before and after a freeze thaw (following several weeks storage of the affinity purified elute at -20°C) revealed that 94% of the protein was recovered using the glycerol-containing buffer, but only 68% of the protein was recovered when the buffer lacking glycerol was employed. This demonstrates that glycerol enhanced the solubility of the pHisBot protein in this low pH buffer when the eluted protein was stored at freezing temperatures (e.g., -20°C). Neutralization of pH by addition of NaH<sub>2</sub>PO<sub>4</sub> buffer did not result in obvious protein precipitation.

It was determined that quantitative binding of pHisBot protein using the batch format occurred after 3 hrs (Figure 30), but not after 1 hr of binding at 4°C (the resin was stirred during binding). Figure 30 depicts a Coomassie blue stained SDS-PAGE gel (7.5% acrylamide) containing samples of proteins isolated during the purification of pHisBot protein from lysate prepared from the BL21(DE3)pLysS host. Each lane was loaded with 5 µl of protein sample mixed with 5 µl of 2X sample buffer and processed as described in Example 22b. Lane 1 contains high molecular weight protein markers (BioRad). Lanes 2 and 3 contain protein eluted from the Ni-NTA resin. Lane 4 contains soluble protein after a 3 hr batch incubation with the Ni-NTA resin. Lanes 5 and 6 contain soluble and total protein, respectively. Figure 30 demonstrates that the pHisBot protein is completely soluble [compare Lanes 5 and 6 which show that a similar amount of the 50 kD pHisBot protein is seen in both: if a substantial amount (greater than 20%) of the pHisBot protein were partially insoluble in the host cell, more pHisBot protein would be seen in lane 6 (total protein) as compared to lane 5 (soluble protein)]. Figure 30 also demonstrates that the pHisBot protein is

completely removed from the lysate after batch absorption with the Ni-NTA resin for 3 hours (compare Lanes 4 and 5).

The reported high affinity interaction of the Ni-NTA resin with His-tagged proteins ( $K_d = 1 \times 10^{-13}$  at pH 8.0) suggested that it should be possible to manipulate the resin-protein complexes without significant release of the bound protein. Indeed, it was determined that after the recombinant protein was bound to the Ni-NTA resin, the resin-pHisBot protein complex was highly stable and remained bound following repeated rounds of centrifugation of the resin for 2 min at 1600 x g. When this centrifugation step was performed in a 50 ml tube (Falcon), a tight resin pellet formed. This allowed the removal of spent soluble lysate by pouring off the supernatant followed by resuspension of the pellet in wash buffer. Further washes can be performed by centrifugation. The ability to perform additional washes permits the development of protocols for batch absorption of large volumes of lysate with removal of the lysate being performed simply by centrifugation following binding of the recombinant protein to the resin.

A simplified, integrated purification protocol was developed as follows. A soluble lysate was made by resuspending the induced cell pellet in binding buffer [50 mM  $\text{NaH}_2\text{PO}_4$ , 0.5 M NaCl, 60 mM imidazole (pH 8.0)], sonicating 4 x 20 sec and centrifuging for 20 min at 10,000 x g. NP-40 was added to 0.1% and Ni-NTA resin (equilibrated in binding buffer) was added. Eight milliliters of a 1:1 slurry (resin:binding buffer) was used per liter of starting culture. The mixture was stirred for 3 hrs at 4°C. The slurry was poured into a column having a 1 cm internal diameter (BioRad), washed with binding buffer containing 0.1% NP40, then binding buffer until baseline was established (these steps may alternatively be performed by centrifugation of the resin, resuspension in binding buffer containing NP40 followed by centrifugation and resuspension in binding buffer). Imidazole was removed by washing the resin with 50 mM  $\text{NaH}_2\text{PO}_4$ , 0.3M NaCl (pH 7.0). Protein bound to the resin was eluted using the same buffer (50 mM  $\text{NaH}_2\text{PO}_4$ , 0.3M NaCl) having a reduced pH (pH 3.5-4.0).

A pilot purification was performed following this protocol and yielded 18 mg/liter affinity-purified pHisBot. The pHisBot protein was greater than 90% pure as estimated by Coomassie staining of an SDS-PAGE gel. This represents the highest observed yield of soluble affinity-purified pHisBot protein and this protocol eliminates the need for separate imidazole-containing binding and wash buffers. In addition to providing a simplified and efficient protocol for the affinity purification of recombinant pHisBot protein, the above

results provide a variety of purification conditions under which pHisBot protein can be isolated.

#### EXAMPLE 26

##### The pHisBot Protein Is An Effective Immunogen

In Example 23 it was demonstrated that neutralizing antibodies are generated in mouse serum after nasal immunization with the pMBot protein. However, the pMBot protein was found to copurify with significant amounts of endotoxin which could not be easily removed. The pHisBot protein, in contrast, could be isolated free of significant endotoxin contamination making pHisBot a superior candidate for vaccine production. To further assess the suitability of pHisBot as a vaccine, the immunogenicity of the pHisBot protein was determined and a comparison of the relative immunogenicity of pMBot and pHisBot proteins in mice was performed as follows.

Two groups of eight BALBc mice were immunized with either pMBot protein or pHisBot protein using Gerbu GMDP adjuvant (CC Biotech). pMBot protein (in PBS containing 10 mM maltose) or pHisBot protein (in 50 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.3 M NaCl, 10% glycerol, pH 4.0) was mixed with Gerbu adjuvant and used to immunize mice. Each mouse received an IP injection of 100 µl antigen/adjuvant mix (50 µg antigen plus 1 µg adjuvant) on day 0. Mice were boosted as described above with the exception that the route of administration was IM on day 14 and 28. The mice were bled on day 77 and anti-*C. botulinum* Type A toxoid titers were determined using serum collected from individual mice in each group (as described in Example 23). The results are shown in Table 41.

TABLE 41

Anti-*C. botulinum* Type A Toxoid Serum IgG Titers In Individual Mice Immunized With pMBot or pHisBot Protein

Mouse #	Preimmune <sup>1</sup>				pMBot <sup>2</sup>				pHisBot <sup>2</sup>			
	Sample Dilution				Sample Dilution				Sample Dilution			
	1:50	1:250	1:1250	1:6250	1:50	1:250	1:1250	1:6250	1:50	1:250	1:1250	1:6250
1					0.678	0.190	0.055	0.007	1.574	0.799	0.320	0.093
2					1.161	0.931	0.254	0.075	1.513	0.829	0.409	0.134
3					1.364	0.458	0.195	0.041	1.596	1.028	0.453	0.122
4					1.622	1.189	0.334	0.067	1.552	0.840	0.348	0.090
5					1.612	1.030	0.289	0.067	1.629	1.580	0.895	0.233
6					0.913	0.242	0.069	0.013	1.485	0.952	0.477	0.145
7					0.910	0.235	0.058	0.014	1.524	0.725	0.269	0.069
8					0.747	0.234	0.058	0.014	1.274	0.427	0.116	0.029
Mean Titer	0.048	0.021	0.011	0.002	1.133	0.564	0.164	0.037	1.518	0.896	0.411	0.114

<sup>1</sup>The preimmune sample represents the average from 2 sets of duplicate wells containing serum from a individual mouse immunized with recombinant *Staphylococcus enterotoxin B* (SEB) antigen. This antigen is immunologically unrelated to *C. botulinum* toxin and provides a control serum

<sup>2</sup>Average of duplicate wells

The results shown above in Table 41 demonstrate that both the pMBot and pHisBot proteins are immunogenic in mice as 100% of the mice (8/8) in each group seroconverted from non-immune to immune status. The results also show that the average titer of anti-*C. botulinum* Type A toxoid IgG is 2-3 fold higher after immunization with the pHisBot protein relative to immunization with the pMBot protein. This suggests that the pHisBot protein may be a superior immunogen to the pMBot protein.

### EXAMPLE 27

#### Immunization With The Recombinant pHisBot Protein Generates Neutralizing Antibodies

The results shown in Example 26 demonstrated that both the pHisBot and pMBot proteins were capable of inducing high titers of anti-*C. botulinum* type A toxoid-reactive antibodies in immunized hosts. The ability of the immune sera from mice immunized with either the pHisBot or pMBot proteins to neutralize *C. botulinum* type A toxoid *in vivo* was determined using the mouse neutralization assay described in Example 23b.

The two groups of eight BALBc mice immunized with either pMBot protein or pHisBot protein in Example 26 were boosted again one week after the bleeding on day 77. The boost was performed by mixing pMBot protein (in PBS containing 10 mM maltose) or pHisBot protein (in 50 mM NaHPO<sub>4</sub>, 0.3 M NaCl, 10% glycerol, pH 4.0) with Gerbu  
5 adjuvant as described in Example 26. Each mouse received an IP injection of 100 µl antigen/adjuvant mix (50 µg antigen plus 1 µg adjuvant). The mice were bled 6 days after this boost and the serum from mice within a group was pooled. Serum from preimmune mice was also collected (this serum is the same serum described in the footnote to Table 41).

The presence of neutralizing antibodies in the pooled or preimmune serum was  
10 detected by challenging mice with 5 LD<sub>50</sub> units of type A toxin mixed with 100 µl of pooled serum. The challenge was performed by mixing (per mouse to be injected) 100 µl of serum from each pool with 100 µl of purified type A toxin standard (50 LD<sub>50</sub>/ml prepared as described in Example 23b) and 500 µl of gel-phosphate. The mixtures were incubated for 30 min at room temperature with occasional mixing. Each of four mice were injected IP with  
15 the mixtures (0.7 ml/mouse). The mice were observed for signs of botulism for 72 hours. Mice receiving toxin mixed with serum from mice immunized with either the pHisBot or pMBot proteins showed no signs of botulism intoxication. In contrast, mice receiving preimmune serum died in less than 24 hours.

These results demonstrate that antibodies capable of neutralizing *C. botulinum* type A  
20 toxin are induced when either of the recombinant *C. botulinum* C fragment proteins pHisBot or pMBot are used as immunogens.

### EXAMPLE 28

#### Cloning And Expression Of The C Fragment of *C. botulinum* 25 Serotype A Toxin In *E. coli* Utilizing A Native Gene Fragment

In Example 22 above, a synthetic gene was used to express the C fragment of *C. botulinum* serotype A toxin in *E. coli*. The synthetic gene replaced non-preferred (*i.e.*, rare) codons present in the C fragment gene with codons which are preferred by *E. coli*. The  
30 synthetic gene was generated because it was been reported that genes which have a high A/T content (such as most clostridial genes) creates expression difficulties in *E. coli* and yeast. Furthermore, LaPenotiere *et al.* suggested that problems encountered with the stability (non-fusion constructs) and solubility (MBP fusion constructs) of the C fragment of *C. botulinum*



serotype A toxin when expressed in *E. coli* was most likely due to the extreme A/T richness of the native *C. botulinum* serotype A toxin gene sequences (LaPenotiere, *et al.*, *supra*).

In this example, it was demonstrated that successful expression of the C fragment of *C. botulinum* type A toxin gene in *E. coli* does not require the elimination of rare codons (i.e., there is no need to use a synthetic gene). This example involved a) the cloning of the native C fragment of the *C. botulinum* serotype A toxin gene and construction of an expression vector and b) a comparison of the expression and purification yields of *C. botulinum* serotype A C fragments derived from native and synthetic expression vectors.

a) **Cloning Of The Native C Fragment Of The *C. botulinum* Serotype A Toxin Gene And Construction Of An Expression Vector**

The serotype A toxin gene was cloned from *C. botulinum* genomic DNA using PCR amplification. The following primer pair was employed: 5'-CGCCATGGCTAG ATTATTATCTACATTTAC-3' (5' primer, *Nco*I site underlined: SEQ ID NO:29) and 5'-GCAAGCTTCTTGACAGACTCATGTAG-3' (3' primer, *Hind*III site underlined: SEQ ID NO:30). *C. botulinum* type A strain was obtained from the American Type Culture Collection (ATCC#19397) and grown under anaerobic conditions in Terrific broth medium. High molecular-weight *C. botulinum* DNA was isolated as described in Example 11. The integrity and yield of genomic DNA was assessed by comparison with a serial dilution of uncut lambda DNA after electrophoresis on an agarose gel.

The gene fragment was cloned by PCR utilizing a proofreading thermostable DNA polymerase (native *Pfu* polymerase). PCR amplification was performed using the above primer pair in a 50 $\mu$ l reaction containing 10mM Tris-HCl (pH 8.3), 50mM KCl, 1.5mM MgCl<sub>2</sub>, 200 $\mu$ M each dNTP, 0.2 $\mu$ M each primer, and 50ng *C. botulinum* genomic DNA. Reactions were overlaid with 100 $\mu$ l mineral oil, heated to 94°C 4 min. 0.5 $\mu$ l native *Pfu* polymerase (Stratagene) was added, and thirty cycles comprising 94°C for 1 min, 50°C for 2 min, 72°C for 2 min were carried out followed by 10 min at 72°C. An aliquot (10 $\mu$ l) of the reaction mixture was resolved on an agarose gel and the amplified native C fragment gene was gel purified using the Prep-A-Gene kit (BioRad) and ligated to pCRScript vector DNA (Stratagene). Recombinant clones were isolated and confirmed by restriction digestion, using standard recombinant molecular biology techniques [Sambrook *et al.* (1989), *supra*]. In addition, the sequence of approximately 300 bases located at the 5' end of the C fragment

coding region were obtained using standard DNA sequencing methods. The sequence obtained was identical to that of the published sequence.

An expression vector containing the native *C. botulinum* serotype A C fragment gene was created by ligation of the *NcoI*-*HindIII* fragment containing the C fragment gene from the pCRScript clone to *NheI*-*HindIII* restricted pETHisa vector (Example 18b). The *NcoI* and *NheI* sites were filled in using the Klenow enzyme prior to ligation; these sites were thus blunt-end ligated together. The resulting construct was termed pHisBotA (native). pHisBotA (native) expresses the *C. botulinum* serotype A C fragment with a his-tagged N terminal extension which has the following sequence:

MetGlyHisHisHisHisHisHisHisHisHisHisSerSerGlyHisIleGluGlyArgHisMetAla (SEQ ID NO:24), where the underlining represents amino acids encoded by the *C. botulinum* C fragment gene (this N terminal extension contains the recognition site for FactorXa protease, shown in italics, which can be employed to removed the polyhistidine tract from the N-terminus of the fusion protein). The pHisBot (native) construct expresses the identical protein as the pHisBot construct (Ex. 24c: herein after the pHisBotA) which contains the synthetic gene.

The predicted DNA sequence encoding the native *C. botulinum* serotype A C fragment gene contained within pHisBotA (native) is listed in SEQ ID NO:31 [the start of translation (ATG) is located at nucleotides 108-110 and the stop of translation (TAA) is located at nucleotides 1494-1496 in SEQ ID NO:31] and the corresponding amino acid sequence is listed in SEQ ID NO:26 (*i.e.*, the same amino acid sequence as that produced by pHisBotA containing synthetic gene sequences).

#### **b) Comparison Of The Expression And Purification Yields Of *C. botulinum* Serotype A C Fragments Derived From Native And Synthetic Expression Vectors**

Recombinant plasmids containing either the native or the synthetic *C. botulinum* serotype A C fragment genes were transformed into *E. coli* strain BI21(DE3) pLysS and protein expression was induced in 1 liter shaker flask cultures. Total protein extracts were isolated, resolved on SDS-PAGE gels and *C. botulinum* C fragment protein was identified by Western analysis utilizing a chicken anti-*C. botulinum* serotype A toxoid antiserum as described in Example 22.

Briefly, 1 liter (2XYT + 100 µg/ml ampicillin and 34 µg/ml chloramphenicol) cultures of bacteria harboring either the pHisBotA (synthetic) or pHisBotA (native) plasmids in the BI21(DE3) pLysS strain were induced to express recombinant protein by addition of IPTG to 1mM. Cultures were grown at 30-32°C. IPTG was added when the cell density reached an OD<sub>600</sub> 0.5-1.0 and the induced protein was allowed to accumulate for 3-4 hrs after induction.

The cells were cooled for 15 min in a ice water bath and then centrifuged for 10 min at 5000 rpm in a JA10 rotor (Beckman) at 4°C. The cell pellets were resuspended in a total volume of 40 mls 1X binding buffer (40 mM imidazole, 0.5 M NaCl, 50 mM NaPO<sub>4</sub>, pH 8.0), transferred to two 50 ml Oakridge tubes and frozen at -70°C for at least 1 hr. The tubes were then thawed and the cells were lysed by sonication (using four successive 20 second bursts) on ice. The suspension was clarified by centrifugation 20-30 min at 9,000 rpm (10,000g) in a JA-17 rotor. The soluble lysate was batch absorbed to 7 ml of a 1:1 slurry of NiNTA resin:binding buffer by stirring 2-4 hr at 4°C. The slurry was centrifuged for 1 min at 500g in 50 ml tube (Falcon), resuspended in 5 mls binding buffer and poured into a 2.5 cm diameter column (BioRad). The column was attached to a UV monitor (ISCO) and the column was washed with binding buffer until a baseline was established. Imidazole was removed by washing with 50mM NaPO<sub>4</sub>, 0.3 M NaCl, 10% glycerol, pH 7.0 and bound protein was eluted using 50mM NaPO<sub>4</sub>, 0.3 M NaCl, 10% glycerol, pH 3.5-4.0.

The eluted proteins were stored at 4°C. Samples of total, soluble, and eluted proteins were resolved by SDS-PAGE. Protein samples were prepared for electrophoresis by mixing 1µl total (T) or soluble (S) protein with 4 µl PBS and 5 µl 2X SDS-PAGE sample buffer, or 5 µl eluted (E) protein and 5 µl 2X SDS-PAGE sample buffer. The samples were heated to 95°C for 5 min, then cooled and 5 or 10 µls were loaded on 12.5% SDS-PAGE gels. Broad range molecular weight protein markers (BioRad) were also loaded to allow the MW of the identified fusion proteins to be estimated. After electrophoresis, protein was detected either generally by staining gels with Coomassie blue, or specifically, by blotting to nitrocellulose for Western blot detection of specific immunoreactive protein.

For Western blot analysis, the gels were blotted, and protein transfer was confirmed by Ponceau S staining as described in Example 22. After blocking the blots for 1 hr at room temperature in blocking buffer (PBST and 5% milk), 10 ml of a 1/500 dilution of an anti-*C. botulinum* toxin A IgY PEG prep (Ex. 3) in blocking buffer was added and the blots were incubated for an additional hour at room temperature. The blots were washed and developed using a rabbit anti-chicken alkaline phosphatase conjugate (Boehringer Mannheim) as the

secondary antibody as described in Ex. 22. This analysis detected *C. botulinum* toxin A-reactive proteins in the pHisBotA (native and synthetic) protein samples (corresponding to the predicted full length proteins identified by Coomassie staining).

A gel containing proteins expressed from the pHisBot and pHisBot (native) constructs during various stages of purification and stained with Coomassie blue is shown in Figure 31. In Figure 31, lanes 1-4 and 9 contain proteins expressed by the pHisBotA construct (*i.e.*, the synthetic gene) and lanes 5-8 contain proteins expressed by the pHisBotA (native) construct. Lanes 1 and 5 contain total protein extracts; lanes 2 and 6 contain soluble protein extracts; lanes 3 and 7 contain proteins which flowed through the NiNTA columns; lanes 4, 8 and 9 contain protein eluted from the NiNTA columns and lane 10 contains molecular weight markers.

The above purification resulted in a yield of 3 mg (native gene) or 11 mg (synthetic gene) of affinity purified protein from a 1 liter starting culture, of which at least 90-95% of the protein was a single band of the predicted MW (50kd) and immunoreactivity for recombinant *C. botulinum* serotype A C fragment protein. Other than the level of expression, no difference was observed between the native and the synthetic gene expression systems.

These results demonstrate that soluble *C. botulinum* serotype A C fragment protein can be expressed in *E. coli* and purified utilizing either native or synthetic gene sequences.

## EXAMPLE 29

### Generation Of Neutralizing Antibodies Using A Recombinant

#### *C. botulinum* Serotype A C Fragment Protein Containing A Six Residue His-Tag

In Example 27, neutralizing antibodies were generated utilizing the pHisBotA protein, which contains a histidine-tagged N-terminal extension comprising 10 histidine residues. To determine if the generation of neutralizing antibodies is dependent on the presence of this particular his-tag, a protein containing a shorter N-terminal extension (comprising 6 histidine residues) was produced and tested for the ability to generate neutralizing antibodies. This example involved a) the cloning and expression of the p6HisBotA(syn) protein and b) the generation and characterization of hyperimmune serum.

a) **Cloning And Expression Of The p6HisBotA(syn) Protein**

The p6HisBotA(syn) construct was generated as described below: the term "syn" designates the presence of synthetic gene sequences. This construct expresses the C fragment of the *C. botulinum* serotype A toxin with a histidine-tagged N terminal extension having the following sequence: MetHisHisHisHisHisHisMetAla (SEQ ID NO:32); the amino acids encoded by the botulinal C fragment gene are underlined and the vector encoded amino acids are presented in plain type.

6XHis oligonucleotides [5'-TATGCATCACCATCACCATCA-3' (SEQ ID NO:33) and 5'-CATGTGATGGTGATGGTGATGCA-3' (SEQ ID NO:34) were annealed as follows. One microgram of each oligonucleotide was mixed in total of 20 µl 1X reaction buffer 2 (NEB) and the mixture was heated at 70°C for 5 min and then incubated at 42°C for 5 min. The annealed oligonucleotides were then ligated with gel purified *NdeI/HindIII* cleaved pET23b (T7 promoter) or pET21b (T7lac promoter) DNA and the gel purified *NcoI/HindIII* *C. botulinum* serotype A C fragment synthetic gene fragment derived from pAlterBot (Ex. 22). Recombinant clones were isolated and confirmed by restriction digestion. The DNA sequence encoding the 6X his-tagged BotA protein contained within p6HisBotA(syn) is listed in SEQ ID NO:35. The amino acid sequence of the p6XHisBotA protein is listed in SEQ ID NO:36.

The resulting recombinant p6XHisBotA plasmid was transformed into the BL21(DE3) pLysS strain, and 1 liter cultures were grown, induced and harvested as described in Example 28. His-tagged protein was purified as described in Example 28, with the following modifications. The binding buffer (BB) contained 5 mM imidazole rather than 40 mM imidazole and NP40 was added to the soluble lysate to a final concentration of 0.1%. The bound material was washed on the column with BB until the baseline was established, then the column was washed successively with BB+20 mM imidazole and BB+40 mM imidazole. The column was eluted as described in Example 28.

In the case of the pET23-derived expression system, high level expression of insoluble 6HisBotA protein was induced. The pET21-derived vector expressed lower levels of soluble protein that bound the NiNTA resin and eluted in the 40 mM imidazole wash rather than during the low pH elution. These results (*i.e.*, low level expression of a soluble protein) are consistent with the results obtained with pHisBotA protein (Ex. 25); the pHisBotA construct, like the pET21-derived vector, contains the T7lac rather than T7 promoter.

The 6HisBotA protein thus elutes under less stringent conditions than the 10X histidine-containing pHisBot protein (100-200 mM imidazole; Ex. 25) presumably due to the

reduction in the length of the his-tag. The eluted protein was of the predicted size [*i.e.*, slightly reduced in comparison to pHisBotA protein].

**b) Generation And Characterization Of Hyperimmune Serum**

5 Eight BALBc mice were immunized with purified 6HisBotA protein using Gerbu GMDP adjuvant (CC Biotech). The 40 mM imidazole elution was mixed with Gerbu adjuvant and used to immunize mice. Each mouse received a subcutaneous injection of 100  $\mu$ l antigen/adjuvant mix (12  $\mu$ g antigen + 1  $\mu$ g adjuvant) on day 0. Mice were subcutaneously boosted as above on day 14 and bled on day 28. Control mice received pHisBotB protein  
10 (prepared as described in Ex. 35 below) in Gerbu adjuvant.

Anti-*C. botulinum* serotype A toxoid titers were determined in serum from individual mice from each group using the ELISA described in Example 23a with the exception that the initial testing serum dilution was 1:100 in blocking buffer containing 0.5% Tween 20, followed by serial 5-fold dilutions into this buffer. The results of the ELISA demonstrated  
15 that seroconversion (relative to control mice) occurred in all 8 mice.

The ability of the anti-*C. botulinum* serotype A C fragment antibodies present in serum from the immunized mice to neutralize native *C. botulinum* type A toxin was tested using the mouse neutralization assay described in Example 23b. The amount of neutralizing antibodies present in the serum of the immunized mice was determined using serum antibody titrations.  
20 The various serum dilutions (0.01 ml) were mixed with 5 LD<sub>50</sub> units of *C. botulinum* type A toxin and the mixtures were injected IP into mice. The neutralizations were performed in duplicate. The mice were then observed for signs of botulism for 4 days. Undiluted serum was found to protect 100% of the injected mice while the 1:10 diluted serum did not. This corresponds to a neutralization titer of 0.05-0.5 IU/ml.

25 These results demonstrate that neutralizing antibodies were induced when the 6HisBotA protein was utilized as the immunogen. Furthermore, these results demonstrate that seroconversion and the generation of neutralizing antibodies does not depend on the specific N terminal extension present on the recombinant *C. botulinum* type A C fragment proteins.

**EXAMPLE 30****Construction Of Vectors For The Expression Of His-Tagged  
*C. botulinum* Type A Toxin C Fragment Protein Using the Synthetic Gene**

5 A number of expression vectors were constructed which contained the synthetic *C. botulinum* type A toxin C fragment gene. These constructs vary as to the promoter (T7 or T7lac) and repressor elements (lacIq) present on the plasmid. The T7 promoter is a stronger promoter than is the T7lac promoter. The various constructs provide varying expression levels and varying levels of plasmid stability. This example involved a) the construction of  
10 expression vectors containing the synthetic *C. botulinum* type A C fragment gene and b) the determination of the expression level achieved using plasmids containing either the kanamycin resistance or the ampicillin resistance genes in small scale cultures.

15 **a) Construction Of Expression Vectors Containing The  
Synthetic *C. botulinum* Type A C Fragment Gene**

Expression vectors containing the synthetic *C. botulinum* type A C fragment gene were engineered to utilize the kanamycin resistance rather than the ampicillin resistance gene. This was done for several reasons including concerns regarding the presence of residual ampicillin in recombinant protein derived from plasmids containing the ampicillin resistance gene. In  
20 addition, ampicillin resistant plasmids are more difficult to maintain in culture: the  $\beta$ -lactamase secreted by cells containing ampicillin resistant plasmids rapidly degrades extracellular ampicillin, allowing the growth of plasmid-negative cells.

A second altered feature of the expression vectors is the inclusion of lacIq gene in the plasmid. This repressor lowers expression from lac regulated promoters (the chromosomally  
25 located, lactose regulated T7 polymerase gene and the plasmid located T7lac promoter). This down regulates uninduced protein expression and can enhance the stability of recombinant cell lines. The final alteration to the vectors is the inclusion of either the T7 or T7lac promoters that drive high or moderate level expression of recombinant protein, respectively.

30 The expression plasmids were constructed as follows. In all cases, the protein expressed is the pHisBotA(syn) protein previously described, and the only differences between constructs is the alteration of the various regulatory elements described above.

i) **Construction Of pHisBotA(syn) kan T7lac**

The pHisBotA(syn) kan T7lac construct was made by inserting the *SapI/XhoI* fragment containing the *C. botulinum* type A C fragment from pHisBotA(syn) into pET24 digested with *SapI/XhoI* (Novagen; fragment contains kan gene and origin of replication). The desired  
5 construct was selected for kanamycin resistance and confirmed by restriction digestion.

ii) **Construction Of pHisBotA(syn) kan lacIq T7lac**

The pHisBotA(syn) kan lacIq T7lac construct was made by inserting the *XbaI/HindIII* fragment containing the *C. botulinum* type A C fragment from pHisBotA(syn)kanT7lac into  
10 the pET24a vector digested with *XbaI/HindIII*. The resulting construct was confirmed by restriction digestion.

iii) **Construction Of pHisBotA(syn) kan lacIq T7**

The pHisBotA(syn) kan lacIq T7 construct was made by inserting the *XbaI/HindIII* fragment containing the *C. botulinum* type A C fragment from pHisBotA(syn) kan lacIq T7lac  
15 into *XbaI/HindIII*-digested pHisBotB(syn) kan lacIq T7 (described in Ex 37c below). The resulting construct was confirmed by restriction digestion.

b) **Determination Of The Expression Level Achieved Using  
20 Plasmids Containing Either The Kanamycin Resistance Or  
The Ampicillin Resistance Genes In Small Scale Cultures**

One liter cultures of pHisBotA(syn) kan T7lac/B121(DE3)pLysS and pHisBotA(syn)  
amp T7lac/B121(DE3)pLysS [this is the previously designated pHisBotA(syn) construct] were  
grown, induced and his-tagged proteins were purified as described in Example 28. No  
25 differences in yield or protein integrity/purity were observed.

These results demonstrate that the antigen induction levels from expression constructs were not affected by the choice of ampicillin versus kanamycin antibiotic resistance genes.



**EXAMPLE 31****Fermentation Of Cells Expressing Recombinant Botulinal Proteins****a) Fermentation Culture Of Cells Expressing Recombinant Botulinal Proteins**

Fermentation cultures were grown under the following conditions which were optimized for growth of the BL21(DE3) strains containing pET derived expression vectors. An overnight 1 liter feeder culture was prepared by inoculating of 1 liter media (in a 2L shaker flask) with a fresh colony grown on an LB kan plate. The feeder culture contained: 600 mls nitrogen source [20 gm yeast extract (BBL) and 40 gm tryptone (BBL)/600 mls], 200 mls 5X fermentation salts (per liter: 48.5 gm  $K_2HPO_4$ , 12 gm  $NaH_2PO_4 \cdot H_2O$ , 5 gm  $NH_4Cl$ , 2.5 gm  $NaCl$ ), 180 mls  $dH_2O$ , 20 mls 20% glucose, 2 mls 1 M  $MgSO_4$ , 5 mls 0.05M  $CaCl_2$ , and 4 mls of a 10 mg/ml kanamycin stock. All solutions were sterilized by autoclaving, except the kanamycin stock which was filter sterilized.

An aliquot (5 ml) of the feeder culture broth was removed prior to inoculation, and grown for 2 days at 37°C as a culture broth sterility control. Growth was not observed in this control culture in any of the fermentations performed.

The inoculated feeder culture was grown for 12-15 hrs (ON) at 30-37°C. Care was taken to prevent oversaturation of this culture. The saturated feeder culture was added to 10L of fermentation media in fermenter (BiofloIV, New Brunswick Scientific, Edison, NJ) as follows. The fermenter was sterilized 120 min at 121°C with  $dH_2O$ . The sterile water was removed, and fermentation media added as follows: 6 liters nitrogen source, 2 liters 5X fermentation salts, 2 liters 2% glucose, 20 mls 1 M  $MgSO_4$ , 50 mls 0.05 M  $CaCl_2$ , 2.5-3.5 mls Macol P 400 antifoam (PPG Industries Inc., Gurnee, IL), 40 mls 10mg/ml kanamycin and 10 mls trace elements (8 gm  $FeSO_4 \cdot 7H_2O$ , 2 gm  $MnSO_4 \cdot H_2O$ , 2 gm  $AlCl_3 \cdot 6H_2O$ , 0.8 gm  $CoCl_2 \cdot 6H_2O$ , 0.4 gm  $ZnSO_4 \cdot 7H_2O$ , 0.4 gm  $Na_2MoO_4 \cdot 2H_2O$ , 0.2 gm  $CuCl_2 \cdot 2H_2O$ , 0.2 gm  $NiCl_2$ , 0.1 gm  $H_3BO_3$ /200mls 5 M HCl). All solutions were sterilized by autoclaving, except the kanamycin stock which was filter sterilized. Fermentation media was prewarmed to 37°C before the addition of the feeder culture.

After the addition of the feeder culture, the culture was fermented at 37°C, 400 rpm agitation, and 10 l/min air sparging. The  $DO_2$  control was set to 20% PID and dissolved oxygen levels were controlled by increasing the rate of agitation from 400-850 rpm under  $DO_2$  control.  $DO_2$  levels were maintained at greater than or equal to 20% throughout the

entire fermentation. When agitation levels reached 500-600 rpm the temperature was lowered to 30°C to reduce the oxygen consumption rate. Culture growth was continued until endogenous carbon sources were depleted. In these fermentations, glucose was depleted first [monitored with a glucose monitoring kit (Sigma)], followed by assimilation of acetate and other acidic carbons [monitored using an acetate test kit (Boehringer Mannheim)]. During the assimilation phase, the pH rose from 6.6-6.8 (starting pH) to 7.4-7.5, at which time the bulk of the remaining carbon source was depleted. This was signaled by a drop in agitation rate (from a maximum of 700-800 rpm) and a rise in DO<sub>2</sub> levels >30%. This corresponds to a OD<sub>600</sub> reading of 18-20/ml. At this point a fed batch mode was initiated, in which a feed solution of 50% glucose was added at a rate of approximately 4 gm glucose/liter/hr. The pH was adjusted to 7.0 by the addition of 25% H<sub>3</sub>PO<sub>4</sub> (approximately 60 mls). Culture growth was continued and reached peak oxygen consumption within the next 3 hrs of growth (while the remaining residual non-glucose carbon sources were assimilated). This phase is characterized by a slow increase in pH, and air sparging was increased to 15L/min, to keep the maximum rpm below 850. Once the residual acidic carbon sources are depleted the agitation rate decreases to 650-750 rpm and the pH begins to drop. pH control was maintained at 7.0 PID by regulated pump addition of a sterile 4M NaOH solution which was consumed at a steady rate for the remainder of the fermentation. Growth was continued at 30°C, and the cultures were grown linearly at a growth rate of 4-7 OD<sub>600</sub> units/hr. to at least 81.5 OD<sub>600</sub> units/ml (>30g/l dry cell weight) without induction. Antifoam (a 1:1 dilution with filter sterilized 100% ethanol) was added as necessary throughout the fermentation to prevent foaming.

During the fed batch mode, glucose was assimilated immediately (concentration in media consistently less than 0.1 gm/liter) and acetate was not produced in significant levels by the pET plasmid/BL21(DE3) cell lines tested (approximately 1 gm/liter at end of fermentation; this is lower than that observed in harvests from shaker flask cultures utilizing the same strains). This was fortuitous, since high levels of acetate has been shown to inhibit induction levels in a variety of expression systems. The above described conditions were found to be highly reproducible between fermentations and utilizing different expression plasmids. As a result, glucose and acetate level monitoring were no longer preformed during fermentation.

**b) Induction Of Fermentation Cultures**

Induction with IPTG (250 mg-10 gms, depending on the expression vector and experiment) was initiated 1-3 hrs after initiation of the glucose feed (30-50 OD<sub>600</sub>/ml). The growth rate after induction was monitored on a hourly basis. Aliquots (5-10 ml) of cells were harvested at the time of induction, and at hourly intervals post-induction. Optical density readings were determined by measuring the absorbance at 600 nm of 10 µl culture in 990 µl PBS versus a PBS control. The growth rate after induction was found to vary depending on the expression system utilized.

**c) Monitoring Of Fermentation Cultures**

Fermentation cultures were monitored using the following control assays.

**i) Colony Forming Ability**

An aliquots of cells were removed from the cultures at each timepoint sampled (uninduced and at various times after induction) were serially diluted in PBS (dilution 1=15 µl cells/3 ml PBS, dilution 2 = 15 µl of dilution 1/3 ml PBS, dilution 3 = 3 or 6 µl of dilution 2/3mls PBS) and 100 µl of dilution 3 was plated on an LB or TSA (trypticase soy agar) plate. The plates were incubated ON at 37°C and then the colonies are counted and scored for macro or micro growth.

**ii) Phenotypic Characterization**

Colonies growing on LB or TSA plates (above) from uninduced and induced timepoints were replica plated onto LB+kan, LB+chloramphenicol (for fermentations utilizing LysS or pACYCGro plasmids), LB+kan+1mM IPTG and LB plates, in this order. The plates were grown 6-8 hrs at 37°C and growth was scored on each plate for a minimum of 40-50 well isolated colonies. The percentage of cells retaining the plasmid at time of induction (*i.e.*, uninduced cultures immediately prior to the addition of IPTG) was determined to be the # colonies LB+Kan (or chloramphenicol) plate/# colonies LB plate X 100%. The percentage of cells with mutated pET plasmids was determined to be the # colonies LB+Kan+IPTG plate/# colonies LB plate X 100%. Colonies on all LB plates were scored morphologically for *E. coli* phenotype as a contamination control. Morphologically detectable contaminant colonies were not detected in any fermentation.

### iii) Recombinant BotA Protein Induction

A total of 10 OD<sub>600</sub> units of cells (e.g., 200 µl of cells at OD<sub>600</sub>=50/ml) were removed from each timepoint sample to a 1.5 ml microfuge tube and pelleted for 2 min at maximum rpm in a microfuge. The pellets were resuspended in 1 ml of 50 mM NaHPO<sub>4</sub>, 0.5 M NaCl, 40mM imidazole buffer (pH 6.8) containing 1 mg/ml lysozyme. The samples were incubated for 20 min at room temperature and stored ON at -70°C. Samples were thawed completely at room temperature and sonicated 2 X 10 seconds with a Branson Sonifier 450 microtip probe at # 3 power setting. The samples were centrifuged for 5 min at maximum rpm in a microfuge.

An aliquot (20 µl) of the protein samples were removed to 20 µl 2X sample buffer, before or after centrifugation, for total and soluble protein extracts, respectively. The samples were heated to 95°C for 5 min, then cooled and 5 or 10 µl were loaded onto 12.5% SDS-PAGE gels. High molecular weight protein markers (BioRad) were also loaded to allow for estimation of the MW of identified fusion proteins. After electrophoresis, protein was detected either generally by staining gels with Coomassie blue, or specifically, by blotting onto nitrocellulose (as described in Ex. 28) for Western blot detection of specific his-tagged proteins utilizing a NiNTA-alkaline phosphatase conjugate exactly as described by the manufacturer (Qiagen).

### iv) Recombinant Antigen Purification

At the end of each fermentation run, 1-10 liters of culture were harvested from the fermenter and the bacterial cells were pelleted by centrifugation at 6000 rpm for 10 min in a JA10 rotor (Beckman). The cell pellets were stored frozen at -70°C or utilized immediately without freezing. Cell pellets were resuspended to 15-20% weight to volume in resuspension buffer (generally 50 mM NaPO<sub>4</sub>, 0.5 M NaCl, 40mM imidazole, pH 6.8) and lysed utilizing either sonication or high pressure homogenization.

For sonication, the resuspension buffer was supplemented with lysozyme to 1 mg/ml, and the suspension was incubated for 20 min, at room temp. The sample was then frozen ON at -70°C, thawed and sonicated 4 X 20 seconds at microtip maximum to reduce viscosity.

For homogenization, the cells were lysed by 2 passes through a homogenizer (Rannie Mini-lab type 8.30 H) at 600 Bar. Cell lysates were clarified by centrifugation for 30 min at 10,000 rpm in a JA10 rotor.

For IDA chromatography, samples were flocculated utilizing polyethyleneimine (PEI) prior to centrifugation. Cell pellets were resuspended in cell resuspension buffer (CRB: 50 mM NaPO<sub>4</sub>, 0.5 M NaCl, 40 mM imidazole, pH 6.8) to create a 20% cell suspension (wet weight of cells/volume of CRB) and cell lysates were prepared as described above (sonication or homogenization). PEI (a 2% solution in dH<sub>2</sub>O, pH 7.5 with HCl) was added to the cell lysate a final concentration of 0.2%, and stirred for 20 min at room temperature prior to centrifugation (8,500 rpm in JA10 rotor for 30 minutes at 4°C). This treatment removed RNA, DNA and cell wall components, resulting in a clarified, low viscosity lysate ("PEI clarified lysate").

His-tagged proteins were purified from soluble lysates by metal-chelate affinity chromatography using either a NiNTA resin (as described in Ex. 28) or an IDA (iminodiacetic acid) resin as described below.

IDA resin affinity purifications were performed utilizing a low pressure chromatography system (ISCO). A 7 ml (small scale) or 70 ml (large scale) Chelating Sepharose Fast Flow (Pharmacia) affinity column was poured; in addition, a second guard column was poured and attached in line with the first column (to capture Ni ions that leached off the affinity column). The columns were washed with 3 column volumes of dH<sub>2</sub>O. The guard column was then removed and the affinity column was washed with 0.3 M NiSO<sub>4</sub> until resistivity was established, then with dH<sub>2</sub>O until the resistivity returned to baseline. The columns were reconnected and equilibrated with cell resuspension buffer (CRB: 50 mM NaPO<sub>4</sub>, 0.5 M NaCl, 40 mM imidazole, pH 6.8). The clarified sample (in CRB) was loaded. Flow rates were 5 ml/min for small scale columns and 20 ml/min for large scale columns. After sample loading, the column was washed with CRB until a baseline established and bound protein was eluted with elution buffer (50 mM NaPO<sub>4</sub>, 0.5 M NaCl, 800 mM imidazole, 20% glycerol, pH 6.8 or 8.0). Protein samples were stored at 4°C or -20°C. The yield of eluted protein was established by measuring the OD<sub>280</sub> of the elutions, with a 1 mg/ml solution of protein assumed to yield an absorbance reading of 2.0.

The IDA columns may be regenerated and reused multiple times (>10). To regenerate the column, the column was washed with 2-3 column volumes of H<sub>2</sub>O, then 0.05 M EDTA until all of the blue/green color was removed followed by a wash with dH<sub>2</sub>O. The IDA columns were sterilized with 0.1 M NaOH (using at least 3 column volumes but not more than 50 minutes contact time with column packing material), then washed with 3 column volumes 0.05 M NaPO<sub>4</sub>, pH 5.0, then dH<sub>2</sub>O and stored at room temperature in 20 % ethanol.

## EXAMPLE 32

## Construction Of A Folding Chaperone Overexpression System

Co-overexpression of the *E. coli* GroEL/GroES folding chaperones in a cell expressing a recombinant foreign protein has been reported to enhance the solubility of some foreign proteins that are otherwise insoluble when expressed in *E. coli* [Gragerouu *et al.* (1992) Proc. Natl. Acad. Sci. USA 89:10344]. The improvement in solubility is thought to be due to chaperone-mediated binding and unfolding of insoluble denatured proteins, thus allowing multiple attempts for productive refolding of recombinant proteins. By overexpressing the chaperones, the unfolding/refolding reaction is driven by excess chaperone, resulting, in some cases, in higher yields of soluble protein.

In this example, a chaperone overexpression system, compatible with pET vector expression systems, was constructed to facilitate testing chaperone-mediated solubilization of *C. botulinum* type A proteins. This example involved the cloning of the GroEL/ES operon and construction of a pLysS-based chaperone hyperexpression system.

The GroEL/GroES operon was PCR amplified and cloned into the pCRScript vector as described in Example 28. The following primer pair was used: 5'-CGCAT ATGAATATTCGTCCATTGCATG-3' (SEQ ID NO:37) [5' primer, start codon of groES gene converted to *NdeI* site (underlined)] and 5'-GGAAAGCTTGCAGGGCAAT *TACATCATG* (SEQ ID NO:38) (3' primer, stop codon of groEL gene italicized, engineered *HindIII* site underlined). Following amplification, the chaperone operon was excised as an *NdeI/HindIII* fragment and cloned into pET23b digested with *NdeI* and *HindIII*. This construction places the Gro operon under the control of the T7 promoter of the pET23 vector. The desired construct was confirmed by restriction digestion.

The T7 promoter-Gro operon-T7 terminator expression cassette was then excised as a *BglII/BspEI* (filled) fragment and cloned into *BamHI* (compatible with *BglII*)/*HindIII* (filled) cleaved pLysS plasmid (this removed the T7 lysozyme gene). The resulting construct was designated pACYCGro, since the plasmid utilizing the pACYC184 origin from the pLysS plasmid. Proper construction was confirmed by restriction digestion.

pACYCGro was transformed into BL21(DE3), cultures were grown and induced with 1 mM IPTG as described in preceding examples. Total and soluble protein extracts were generated from cells removed before and after IPTG induction and were resolved on a 12.5 % SDS-PAGE gel and stained with Coomassie blue. This analysis revealed that high levels of

soluble GroEl and GroES proteins were made in the induced cells. These results demonstrated that the chaperone hyper-expression system was functional.

### EXAMPLE 33

#### Growth Of BotA/pACYCGro Cell Lines In Fermentation Cultures

Induction of BL21(DE3) cells lacking the LysS plasmid which contained BotA expression constructs grown in shaker flask or fermentation culture resulted in the expression of primarily insoluble BotA protein. Fermentation cultures were performed to determine if the simultaneous overexpression of the Gro operon and recombinant *C. botulinum* type A proteins (BotA proteins) resulted in enhanced solubility of the recombinant BotA protein. This example involved the fermentation of pHisBotA(syn)kan lacIq T7lac/pACYCGro BL21(DE3) and pHisBotA(syn)kan lacIq T7/pACYCGro BL21(DE3) cell lines. The fermentations were repeated exactly as described in Example 31. Chloramphenicol (34 µg/ml) was included in the feeder and fermentation cultures.

#### a) Fermentation Of pHisBotA(syn)kan lacIq T7lac/pACYCGro BL21(DE3) Cells

For fermentation of cells containing plasmids comprising the T7lac promoter, induction was with 2 gms IPTG at 1 hr post initiation of glucose feed. The OD<sub>600</sub> was 35 at time of induction, then 48.5, 61.5, 67 at 1-3 hrs post induction. Viable colony counts decreased from 0-3 hr induction [21 (13), 0, 0, 0; dilution 3 utilized 3 µl of dilution 2 cells] with numbers in parenthesis for the indicating microcolonies. Of 28 colonies scored at the time of induction, 23 retained the pHisBotA(syn)kan lacIq T7lac plasmid (kan resistant), 22 contained the chaperone plasmid (chloramphenicol resistant) and no colonies at induction grew on IPTG+Kan plates (no mutations detected). These results were indicative of very strong promoter induction, since colony viability dropped immediately after induction.

Total and soluble extracts were resolved on a 12.5% SDS-PAGE gel and stained with Coomassie. High level induction of Gro chaperones was observed, but very low level expression of soluble BotA protein was observed, increasing from 1 to 4.0 hrs post induction (no expression detected in uninduced cells). The dramatically lower expression of the BotA antigen in the presence of chaperone may be due to promoter occlusion (*i.e.*, the stronger T7 promoter on the chaperone plasmid is preferentially utilized).

**b) Fermentation Of pHisBotA(syn)kan lacIq T7/ pACYCGro  
BL21(DE3) Cells**

A fermentation utilizing the T7-driven BotA expression plasmid was performed. Induction was with 1 gm IPTG at 2 hrs post initiation of glucose feed. The OD<sub>600</sub> was 41 at time of induction, then 51.5, 61.5, 61.5 and 66 at 1-4 hrs post induction. Viable colony counts decreased from 0-4 hrs induction [71, 1 (34), 1 (1), 1, 0; dilution 3 utilized 6 µl dilution 2 cells) with numbers in parenthesis for the uninduced timepoint indicating microcolonies. Of 65 colonies scored at the time of induction, all 65 retained both the pHisBotA(syn)kan lacIq T7 plasmid (kan resistant) and the chaperone plasmid (chloramphenicol resistant) and no colonies at induction grew on IPTG+Kan plates (no mutations detected).

Total and soluble extracts were resolved on a 12.5% SDS-PAGE gel and stained with Coomassie. High level induction of Gro chaperones and moderate level expression of soluble BotA protein was observed, increasing from 1 to 4.0 hrs post induction (no expression detected in uninduced cells).

A PEI-clarified lysate (0.2% final concentration PEI) [850 ml from 130 gm cell pellet (2 liters fermentation harvest)] was purified on a large scale IDA column. A total of 78 mg of protein was eluted. Extracts from the purification were resolved on a 12.5% SDS-PAGE gel and stained with Coomassie. The elution was found to contain an approximately 1:1 mix of BotA/chaperone protein (Figure 32). PEI lysates prepared in this manner were typically 16 OD<sub>280</sub>/ml. This was estimated to be 8 mg protein/ml of lysate (by BCA assay). Thus, the eluted recombinant BotA protein represented 0.55% of the total soluble cellular protein applied to the column.

In Figure 32, lane 1 contains molecular weight markers, lanes 2-9 contain extracts from pHisBotA(syn)kan lacIq T7/pACYCGro/BL21(DE3) cells before or during purification on the IDA column. Lane 2 contains total protein extract; lane 3 contains soluble protein extract; lanes 4 and 5 contain PEI-clarified lysates (duplicates); lanes 6 and 7 contain flow-through from the IDA column (duplicates) and lanes 8 and 9 contain IDA column elute (lane 9 contains 1/10 the amount applied to lane 8).

These results demonstrate, that although the majority of the BotA protein produced was insoluble, 20 mg/liter of soluble recombinant BotA protein can be purified utilizing the pHisBotA(syn)kan lacIq T7/pACYCGro/BL21(DE3) expression system.



**EXAMPLE 34****Purification Of Recombinant BotA Protein From Folding Chaperones**

5 In this example of size exclusion chromatography was used to purify the recombinant BotA protein away from the folding chaperones and imidazole present in the IDA-purified material (Ex. 33).

To enhance the solubility of the recombinant BotA protein during scale-up, the protein was co-expressed with folding chaperones (Ex. 33). As observed with the recombinant BotB protein (Example 40 below), the folding chaperones co-eluted with the recombinant BotA protein during the Ni-IDA purification step. Because the recombinant BotA and BotB proteins have similar molecular weights (about 1/10 the size of the non-reduced folding chaperone) and the imidazole step gradient strategy was unsuccessful in purifying BotB away from the folding chaperone (see Ex. 40), size exclusion chromatography was examined for the ability to purify the recombinant BotA protein away from the folding chaperones.

15 A column (2.5 x 24 cm) containing Sephacryl S-100 HR (Pharmacia) was poured (bed volume = 110 ml). Proteins having molecular weights greater than 100 K are expected to elute in the void volume under these conditions and smaller proteins should be retained by the beads and elute at different times, depending on their molecular weights. To maintain solubility of the purified BotA protein, the Sephacryl column was equilibrated in a buffer having the same salt concentration as the buffer used to elute the BotA protein from the IDA column (*i.e.*, 50 mM sodium phosphate, 0.5 M NaCl, 10% glycerol; all reagents from Mallinkrodt, Chesterfield, MO).

Five milliliters of the IDA-purified recombinant BotA protein (Ex. 33) was filtered through a 0.45  $\mu$  syringe filter, applied to the column and the equilibration buffer was pumped through the column at a flow rate of 1 ml/minute. Eluted proteins were monitored by absorbance at 280 nm and collected either manually or with a fraction collector (BioRad). Appropriate fractions were pooled, if necessary, and the protein was quantitated by absorbance at 280 nm and/or BCA protein assay (Pierce). The isolated peaks were then analyzed by native and/or SDS-PAGE to identify the proteins present and to evaluate purity. The folding chaperone eluted first, followed by the recombinant BotA protein and then the imidazole peak.

30 SDS-PAGE analysis (12.5% polyacrylamide, reduced samples) was used to evaluate the purity of the IDA-purified recombinant BotA protein before and after S-100 purification.

Figure 33 shows the difference in purity before and after the S-100 purification step. In Figure 33, lane 1 contains molecular weight markers (BioRad broad range). Lane 2 shows the IDA-purified recombinant BotA protein preparation, which is contaminated with significant amounts of the folding chaperone. Following S-100 purification, the amount of folding chaperone present in the BotA sample is reduced dramatically (lane 3). Lane 4 contains no protein (*i.e.*, it is a blank lane); lanes 5-8 contain samples of IDA-purified recombinant BotB and BotE proteins and are discussed *infra*.

Endotoxin levels in the S-100 purified BotA preparation were determined using the LAL assay (Associates of Cape Cod) as describe in Example 24. The purified BotA preparation was found to contain 22.7 to 45.5 EU/mg recombinant protein.

These results demonstrate that size exclusion chromatography was successful in purifying the recombinant BotA protein from folding chaperones and imidazole following an initial IDA purification step. Furthermore, these results demonstrate that the S-100 purified BotA protein was substantially free of endotoxin.

### EXAMPLE 35

#### Cloning And Expression Of The C Fragment Of The *C. botulinum* Serotype B Toxin Gene

The *C. botulinum* type B neurotoxin gene has been cloned and sequenced [Whelan *et al.* (1992) Appl. Environ. Microbiol. 58:2345 and Hutson *et al.* (1994) Curr. Microbiol. 28:101]. The nucleotide sequence of the toxin gene derived from the Eklund 17B strain (ATCC 25765) is available from the EMBL/GenBank sequence data banks under the accession number X71343; the nucleotide sequence of the coding region is listed in SEQ ID NO:39. The amino acid sequence of the *C. botulinum* type B neurotoxin derived from the strain Eklund 17B is listed in SEQ ID NO:40. The nucleotide sequence of the *C. botulinum* serotype B toxin gene derived from the Danish strain is listed in SEQ ID NO:41 and the corresponding amino acid sequence is listed in SEQ ID NO:42.

The DNA sequence encoding the native *C. botulinum* serotype B C fragment gene derived from the Eklund 17B strain can be expressed using the pETHisb vector; the resulting coding region is listed in SEQ ID NO:43 and the corresponding amino acid sequence is listed in SEQ ID NO:44. The DNA sequence encoding the native *C. botulinum* serotype B C fragment gene derived from the Danish strain can be expressed using the pETHisb vector; the

resulting coding region is listed in SEQ ID NO:45 and the corresponding amino acid sequence is listed in SEQ ID NO:46. The C fragment region from any strain of *C. botulinum* serotype B can be amplified and expressed using the approach illustrated below using the C fragment derived from *C. botulinum* type B 2017 strain.

5 The *C. botulinum* type B neurotoxin gene is synthesized as a single polypeptide chain which is processed to form a dimer composed of a light and a heavy chain linked via disulfide bonds; the type B neurotoxin has been reported to exist as a mixture of predominately single chain with some double chain (Whelan *et al.*, supra). The 50 kD carboxy-terminal portion of the heavy chain is referred to as the C fragment or the H<sub>C</sub> domain. Expression of  
10 the C fragment of *C. botulinum* type B toxin in heterologous hosts (e.g., *E. coli*) has not been previously reported.

The native C fragment of the *C. botulinum* serotype B toxin gene was cloned and expression constructs were made to facilitate protein expression in *E. coli*. This example involved PCR amplification of the gene, cloning, and construction of expression vectors.

15 The C fragment of the *C. botulinum* serotype B (BotB) toxin gene was cloned using the protocols and conditions described in Example 28 for the isolation of the native BotA gene. The *C. botulinum* type B 2017 strain was obtained from the American Type Culture Collection (ATCC #17843). The following primer pair was used to amplify the BotB gene: 5'-CGCCATGGCTGATACAATACTAATAGAA ATG-3' [5' primer, engineered *Nco*I site underlined (SEQ ID NO:47)] and 5'-GCAAG CTTTATTTCAGTCCACCCTTCATC-3' [3'  
20 primer, engineered *Hind*III site underlined, native gene termination codon italicized (SEQ ID NO:48)]. After cloning into the pCRscript vector, the *Nhe*I(filled)/*Hind*III fragment was cloned into pETHisb vector as described for BotA C fragment gene in Example 28. The resulting construct was termed pHisBotB.

25 pHisBotB expresses the BotB gene sequences under the transcriptional control of the T7 lac promoter and the resulting protein contains an N-terminal 10XHis-tag affinity tag. The pHisBotB expression construct was transformed into BL21(DE3) pLysS competent cells and 1 liter cultures were grown, induced and his-tagged proteins were purified utilizing a NiNTA resin (eluted in low pH elution buffer) as described in Example 28. Total, soluble and  
30 purified proteins were resolved by SDS-PAGE and detected by Coomassie staining and Western blot hybridization utilizing a chicken anti-*C. botulinum* serotype B toxoid primary antibody (generated by immunization of hens using *C. botulinum* serotype B toxoid as described in Example 3). Samples of BotA and BotE C fragment proteins were included on

the gels for MW and immunogenicity comparisons. Strong immunoreactivity to only the BotB protein was detected with the anti-*C. botulinum* serotype B toxoid antibodies. The recombinant BotB protein was expressed at low levels (3 mg/liter) as a soluble protein. The purified BotB protein migrated as a single band of the predicted MW (i.e., ~50kD).

These results demonstrate the cloning of the native *C. botulinum* serotype B C fragment gene, the expression and purification of the recombinant BotB protein as a soluble his-tagged protein in *E. coli*.

### EXAMPLE 36

#### Generation Of Neutralizing Antibodies Using The Recombinant pHisBotB Protein

The ability of the purified pHisBot protein to generate neutralizing antibodies was examined. Nine BALBc mice were immunized with BotB protein (purified as described in Ex. 35) using Gerbu GMDP adjuvant (CC Biotech). The low pH elution was mixed with Gerbu adjuvant and used to immunize mice. Each mouse received a subcutaneous injection of 100 µl antigen/adjuvant mix (15 µg antigen + 1 µg adjuvant) on day 0. Mice were subcutaneously boosted as above on day 14 and bled on day 28. Mice were subsequently boosted 1-2 weeks after bleeding and were then bled on day 70.

Anti-*C. botulinum* serotype B toxoid titers were determined in day 28 serum from individual mice from each group using the ELISA protocol outlined in Example 29 with the exception that the plates were coated with *C. botulinum* serotype B toxoid, and the primary antibody was a chicken anti-*C. botulinum* serotype B toxoid. Seroconversion [relative to control mice immunized with pHisBotE antigen (described below)] was observed with all 9 mice immunized with the purified pHisBotB protein.

The ability of the anti-BotB antibodies to neutralize native *C. botulinum* type B toxin was tested in a mouse-*C. botulinum* neutralization model using pooled mouse serum (see Ex. 23b). The LD<sub>50</sub> of purified *C. botulinum* type B toxin complex (Dr. Eric Johnson, University of Wisconsin, Madison) was determined by a intraperitoneal (IP) method [Schantz and Kautler (1978), *supra*] using 18-22 g female ICR mice. The amount of neutralizing antibodies present in the serum of the immunized mice was determined using serum antibody titrations. The various serum dilutions (0.01 ml) were mixed with 5 LD<sub>50</sub> units of *C. botulinum* type B toxin and the mixtures were injected IP into mice. The neutralizations were performed in duplicate. The mice were then observed for signs of botulism for 4 days. Undiluted serum (day 28 or

day 70) was found to protect 100% of the injected mice while the 1:10 diluted serum did not. This corresponds to a neutralization titer of 0.05-0.5 IU/ml.

These results demonstrate that seroconversion occurred and neutralizing antibodies were induced when the pHisBotB protein was utilized as the immunogen.

### EXAMPLE 37

#### Construction Of Vectors To Facilitate Expression Of His-Tagged BotB Protein In Fermentation Cultures

A number of expression vectors were constructed to facilitate the expression of recombinant BotB protein in large scale fermentation culture. These constructs varied as to the strength of the promoter utilized (T7 or T7lac) and the presence of repressor elements (lacIq) on the plasmid. The resulting constructs varied in the level of expression achieved and in plasmid stability which facilitated the selection of a optimal expression system for fermentation scaleup.

The BotB expression vectors created for fermentation culture were engineered to utilize the kanamycin rather than the ampicillin resistance gene, and contained either the T7 or T7lac promoter, with or without the lacIq gene for the reasons outlined in Example 30.

In all cases, the protein expressed by the various expression vectors is the pHisBot B protein described in Example 35, with the only differences between clones being the alteration of various regulatory elements. Using the designations outlined below, the pHisBotB clone (Ex. 35) is equivalent to pHisBotB amp T7lac.

#### a) Construction Of pHisBotB kan T7lac

pHisBotB kan T7lac was constructed by insertion of the *Bgl*II/*Hind*III fragment of pHisBotB which contains the BotB gene sequences into the pPA1870-2680 kan T7lac vector which had been digested with *Bgl*II and *Hind*III (the pPA1870-2680 kan T7lac vector contains the pET24 kan gene in the pET23 vector, such that no lacIq gene is present). Proper construction of pHisBotB kan T7lac was confirmed by restriction digestion.

**b) Construction Of pHisBotB kan lacIq T7lac**

pHisBotB kan lacIq T7lac was constructed by insertion of the *BglII/HindIII* fragment of pHisBotB which contains the BotB gene sequences into similarly cut pET24a vector. Proper construction of pHisBotB kan lacIq T7lac was confirmed by restriction digestion.

**c) Construction Of pHisBotB kan lacIq T7**

pHisBotB kan lacIq T7 was constructed by inserting the *NdeI/XhoI* fragment from pHisBotE kan lacIq T7lac which contains the BotB gene sequences into similarly cleaved pPA1870-2680 kan lacIq T7 vector (this vector contains the T7 promoter, the same N-terminal his-tag as the Bot constructs, the *C. difficile* toxin A insert, and the kan lacIq genes; this cloning replaces the *C. difficile* toxin A insert with the BotB insert). Proper construction was confirmed by restriction digestion.

Expression of recombinant BotB protein from these expression vectors and purification of the BotB protein is described in Example 38 below.

**EXAMPLE 38****Fermentation And Purification Of Recombinant BotB Protein Utilizing The pHisBotB kan lacIq T7lac, pHisBotB kan T7lac And pHisBotB kan lacIq T7 Vectors**

The pHisBotB kan lacIq T7lac, pHisBotB kan T7lac and BotB kan lacIq T7 constructs [all transformed into the BI21(DE3) strain] were grown in fermentation cultures to determine the utility of the various constructs for large scale expression and purification of soluble BotB protein. All fermentations were performed as described in Example 31.

**a) Fermentation Of pHisBotB kan lacIq T7lac/BI21(DE3) Cells**

The fermentation culture was induced 45 min post start of glucose feed with 1 gm IPTG (final concentration = 0.4 mM). pH was maintained at 6.5 rather than 7.0. The  $OD_{600}$  was 27 at time of induction, then 35, 38, and 40 at 1-3 hrs post induction. Duplicate platings of diluted 1 hr induction samples (dilutions were prepared as described Ex. 31, dilution 3 utilized 3  $\mu$ l of dilution 2 cells) on TSA and LB+kan plates yielded 89 TSA colonies and 81 kan colonies (90% kan resistant).

Total and soluble protein extracts were resolved on a 12.5% SDS-PAGE gel and total protein was detected by staining with Coomassie blue. Low level induction of insoluble Bot

B protein was observed, increasing from 1 to 3 hrs post induction (no expression was detected in uninduced cells).

**b) Fermentation Of pHisBotB kan T7lac/BI21(DE3) Cells**

The fermentation culture was induced 1 hr post start of glucose feed with 2 gm IPTG (final concentration = 0.8 mM). pH was maintained at 6.5 rather than 7.0. The OD<sub>600</sub> was 24.5 at time of induction, then 31.5, 32, and 33 at 1-3 hrs post induction, respectively. Duplicate platings of diluted 0 hr and 2 hr induction samples (dilutions were prepared as described Ex. 31: dilution 3 utilized 3 µl of dilution 2 cells) on TSA and LB+kan plates yielded 32 TSA colonies and 54 kan colonies (all kan resistant) for uninduced cells, and 1 TSA colony and 0 kan colonies 2 hr post induction. These results were indicative of strong induction, since viable counts decreased dramatically 2 hrs post induction.

Total and soluble extracts were resolved on a 10% SDS-PAGE gel and total protein was detected by staining with Coomassie blue. Moderate induction of insoluble BotB protein was observed, increasing from 1 to 3 hrs post induction (no expression was detected in uninduced cells).

**c) Fermentation Of pHisBotB kan lacIq T7/BI21(DE3) Cells**

The fermentation was induced 2 hr post start of glucose feed with 4 gm IPTG (final concentration = 1.6 mM). pH was maintained at 6.5 rather than 7.0. The OD<sub>600</sub> was 45 at time of induction, then 47, 50, and 50 and 55 at 1-4 hrs post induction, respectively. Viable colony counts decreased after induction (96, 1, 1, 2, 3: dilution 3 utilized 3 µl of dilution 2 cells). Of 63 colonies scored at the time of induction, all 63 retaining the BotB plasmid (kan resistant) and no colonies at induction grew on IPTG + Kan plates (no mutations detected).

Total and soluble extracts were resolved on a 12.5% SDS-PAGE gel and total protein was detected by staining with Coomassie blue. Moderate level induction of insoluble BotB protein was observed, increasing from 1 to 4 hrs post induction (lower level expression was detected in uninduced cells, since the T7 rather than T7lac promoter was utilized).

**d) Purification Of pHisBotB Protein From pHisBotB amp T7lac/BI21(DE3) Cells**

Soluble recombinant BotB protein was purified utilizing NiNTA resin from 80 ml of cell lysate generated from cells harvested from a pHisBotB fermentation [using the pHisBotB

amp T7lac/BI21(DE3) strain]. As predicted from the small scale results above, the majority of the induced protein was insoluble. As well, the eluted material was contaminated with multiple *E. coli* contaminant proteins. A Coomassie blue-stained SDS-PAGE gel containing extracts derived from pHisBotB amp T7lac/BI21(DE3) cells before and during purification is shown in Figure 34. In Figure 34, lane 1 contains broad range protein MW markers (BioRad). Lanes 2-5 contain extracts prepared from pHisBotB amp T7lac/BI21(DE3) cells grown in fermentation culture: lane 2 contains total protein; lane 3 contains soluble protein; lane 4 contains protein which did not bind to the NiNTA column (*i.e.*, the flow-through) and lane 5 contains protein eluted from the NiNTA column.

Similar results were obtained using a small scale IDA column utilizing a cell lysate from the pHisBotB kan lacIq T7 fermentation described above. 250 mls of a 20% w/v PEI clarified lysate (50 gms cell pellet) of botB kan lacIq T7/BI21(DE3) cells were purified on a small scale IDA column. The total yield of eluted protein was 21 mg protein (assuming 1 mg/ml solution = 2 OD<sub>280</sub>/ml). When analyzed by SDS-PAGE and Coomassie staining, the BotB protein was found to comprise approximately 50% of the eluted protein with the remainder being a ladder of *E. coli* proteins similar to that observed with the NiNTA purification.

The NiNTA alkaline phosphatase conjugate was utilized to detect his-tagged proteins on a Western blot containing total, soluble, soluble (PEI clarified), soluble (after IDA column) and elution samples from the IDA column purification. The results demonstrated that a small percentage of BotB protein was soluble, that the soluble protein was not precipitated by PEI treatment and was quantitatively bound by the IDA column. Since a 1 liter fermentation harvest yielded a 67.5 gm cell pellet, this indicated that the yield of soluble affinity purified BotB protein from the IDA column was 14 mg/liter.

### EXAMPLE 39

#### Co-Expression Of Recombinant BotB Proteins And Folding Chaperones In Fermentation Cultures

Fermentations were performed to determine if the simultaneous overexpression of folding chaperones (*i.e.*, the Gro operon) and the BotB protein resulted in enhanced solubility of the Bot B protein. This example involved fermentation of the pHisBotBkan lacIq T7lac/pACYCGro BL21(DE3), pHisBotB kan T7lac/pACYCGro BI21(DE3) and pHisBotBkan



lacIq T7/ pACYCGro BL21(DE3) cell lines. Fermentation was carried out as described in Example 31; 34 µg/ml chloramphenicol was included in the feeder and fermentation cultures.

a) **Fermentation Of pHisBotBkan lacIq T7lac/pACYCGro  
BL21(DE3) Cells**

Induction was with 4 gms IPTG at 1 hr 15 min post initiation of the glucose feed. The OD<sub>600</sub> was 38 at time of induction, then 50, 58.5, 62 and 68 at 1-4 hrs post induction. Viable colony counts decreased during induction (24, 0, 0, 2, 0 at 0-4 hr induction: dilution 3 utilized 3 µl of dilution 2 cells). Of 24 colonies scored at the time of induction, 24 retained the BotB plasmid (kan resistant), 24 contained the chaperone plasmid (chloramphenicol resistant) and no colonies at induction grew on IPTG+Kan plates (no mutations detected).

Total and soluble extracts were resolved on 12.5% SDS-PAGE gels and were either stained with Coomassie blue or subjected to Western blotting (his-tagged proteins were detected utilizing the NiNTA-alkaline phosphatase conjugate). This analysis revealed that the Gro chaperones were induced to high levels, but very low level expression of soluble BotB protein was observed, increasing from 1 to 4.0 hrs post induction (no expression detected in uninduced cells, induced protein detected only on Western blot). The dramatically lower expression of BotB protein in the presence of chaperone may be due to promoter occlusion (*i.e.*, the stronger T7 promoter on the chaperone plasmid was preferentially utilized).

b) **Fermentation Of pHisBotB kan T7lac/pACYCGro/BI21(DE3)  
Cells**

Induction was with 4 gms IPTG at 1 hr post initiation of the glucose feed. The OD<sub>600</sub> was 33.5 at time of induction, then 44, 51, 58.5 and 69 at 1-4 hrs post induction. Viable colony counts decreased after 2 hrs induction (43, 65, 74, 0 (70), 0 (70) at 0-4 hr induction: bracketed numbers represent microcolonies; dilution 3 utilized 3 µl of dilution 2 cells). Most colonies at induction retained the BotB plasmid (kan resistant) and the chaperone plasmid (chloramphenicol resistant) and no colonies at induction grew on IPTG+Kan plates (no mutations detected).

Total and soluble extracts were resolved on a 12.5% SDS-PAGE gel and subjected to Western blotting: his-tagged proteins were detected utilizing the NiNTA-alkaline phosphatase conjugate. This analysis revealed that the Gro chaperones were induced to high levels and

low level expression of soluble Bot B protein was observed, increasing from 1 to 4.0 hrs post induction (no expression detected in uninduced cells).

A small scale IDA purification of BotB protein from a 250 ml PEI clarified 15% w/v extract (37.5 gm cell pellet) yielded approximately 12.5 mg protein, of which approximately 50% was BotB protein and 50% was GroEL chaperone (assessed by Coomassie staining of a 10% SDS-PAGE gel). The NiNTA alkaline phosphatase conjugate was utilized to detect his-tagged proteins on a Western blot containing total, soluble, soluble (PEI clarified), soluble (after IDA column) and elution samples from the IDA column purification. The results demonstrated that all of the BotB protein produced by the pHisBotB kan T7lac/pACYCGro/BI21(DE3) cells was soluble; the BotB protein was not precipitated by PEI treatment and was quantitatively bound by the IDA column. Since a 1 liter fermentation harvest yielded a 75 gm cell pellet, this indicated that the yield of soluble affinity purified bot B protein from this fermentation was 12.5 mg/liter. These results also demonstrated that additional purification steps are necessary to separate the chaperone proteins from the BotB protein.

**c) Fermentation Of pHisBotBkan lacIq  
T7/pACYCGro/BL21(DE3) Cells**

Induction was with 4 gms IPTG at 2 hr post initiation of the glucose feed. The OD<sub>600</sub> was 46 at time of induction, then 56, 63, 69 and 71.5 at 1-4 hrs post induction. Viable colony counts decreased after induction (58, 3(5), 3, 0, 0 at 0-4 hr induction; bracketed numbers represent microcolonies; dilution 3 utilized 3 µl of dilution 2 cells). All (53/53) colonies scored at the time of induction retained the BotB plasmid (kan resistant) and the chaperone plasmid (chloramphenicol resistant) and no colonies at induction grew on IPTG+Kan plates (no mutations detected).

Total and soluble extracts were resolved on a 10% SDS-PAGE gels and Western blotted and his-tagged proteins were detected utilizing the NiNTA-alkaline phosphatase conjugate. This analysis revealed that the Gro chaperones were induced to high levels (observed by ponceau S staining), and a much higher expression of soluble Bot B protein (compared to expression in the pHisBotB kan T7lac/pACYCGro fermentation) was observed at all timepoints, including uninduced cells (some increase in BotB protein levels were observed after induction).

A small scale IDA purification of BotB protein from a 100 ml PEI clarified 15% w/v extract (15 gm cell pellet) yielded approximately 40 mg protein, of which approximately 50% was BotB protein and 50% was GroEL chaperone, as assessed by Coomassie staining of a 10% SDS-PAGE gel. The NiNTA alkaline phosphatase conjugate was utilized to detect his-tagged proteins on a Western blot containing total, soluble, soluble (PEI clarified), soluble (after IDA column) and elution samples from the IDA column purification. The results demonstrated that a significant percentage (*i.e.*, ~10-20 %) of BotB protein was soluble, that the solubilized protein was not precipitated by PEI treatment and was quantitatively bound by the IDA column. Since a 10 liter fermentation yielded a 108 gm cell pellet, this indicated that the yield of soluble affinity purified BotB protein from this fermentation was 144 mg/liter.

In a scale up experiment, 2 liters of a 20% w/v PEI clarified lysate of pHisBotB-kan lacIq T7/pACYCGro/BL21(DE3) cells were purified on a large scale IDA column. The purification was performed in duplicate. The total yield of BotB protein was 220 and 325 mgs protein in the two experiments (assuming 1 mg/ml solution = 2.0 OD<sub>280</sub>/ml). This represents 0.7% or 1.0%, respectively, of the total soluble cellular protein (assuming a PEI lysate having a concentration of 8 mg protein/ml and that the eluted material comprises a 1:1 mixture of BotB and folding chaperone). The NiNTA alkaline phosphatase conjugate was utilized to detect his-tagged proteins on a Western blot containing total, soluble, soluble (PEI clarified), soluble (after IDA column) and elution samples from the IDA column purification. These results demonstrated that a significant percentage (*i.e.*, ~10-20 %) of the BotB protein was soluble, that the solubilized protein was not precipitated by PEI treatment and was quantitatively bound by the IDA column. Since a 1 liter fermentation harvest yielded a 108 gm cell pellet, this indicated that the yield of soluble affinity purified BotB protein from the large scale purification was 60 mg or 89 mg/liter. These results also demonstrated that further purification would be necessary to remove the contaminating chaperone protein.

The above results provide methodologies for the purification of soluble BotB protein from fermentation cultures, in a form contaminated predominantly with a single *E. coli* protein (the folding chaperone utilized to enhance solubility). In the next example, methods are provided for the removal of the contaminating chaperone protein.

**EXAMPLE 40****Removal Of Contaminating Folding Chaperone Protein  
From Purified Recombinant *C. botulinum* Type B Protein**

5 In this example size exclusion chromatography and ultrafiltration was used to purify recombinant BotB protein from the folding chaperones and imidazole in IDA-purified material.

To enhance the solubility of the recombinant BotB protein during scale-up, the protein was co-expressed with folding chaperones (see Ex. 39). During the Ni-IDA purification step,  
10 the folding chaperones co-eluted with the BotB protein in 800 mM imidazole; therefore, a second purification step was required to isolate the BotB free of folding chaperones. Lane 3 of Figure 35 contains proteins eluted from an IDA column to which a lysate of pHisBotB kan lacIq T7/pACYCGro/BL21(DE3) cells had been applied; the proteins were resolved on a 4-15% polyacrylamide pre-cast gradient gel (Bio-Rad, Hercules, CA) run under native  
15 conditions and then stained with Coomassie blue. In Figure 35, lanes 1 and 4 contain proteins present in peak 1 and peak 2 from a Sephacryl S-100 column run as described below; lane 2 is blank.

As seen in lane 3 of Figure 35, the IDA-purified sample consists primarily of the folding chaperones and the BotB protein. The fact that the chaperones and the Bot B antigen  
20 appear as two distinct bands under native conditions suggested they were not complexed together and therefore, it should be possible to separate them, using either a gradient of imidazole concentrations or size exclusion methods.

In order to determine whether a gradient of imidazole concentrations could be used to separate the chaperone from the BotB protein, a step gradient using imidazole at 200, 400,  
25 600, and 800 mM in 50 mM sodium phosphate, 0.5 M NaCl and 10 % glycerol, pH 6.8 was applied to an IDA column (containing proteins bound from a lysate of pHisBotB kan lacIq T7/pACYCGro/BL21(DE3) cells). By narrowing the range of imidazole concentrations, it was hoped that the BotB and chaperone proteins would differentially elute at different concentrations of imidazole. Eluted proteins were monitored by absorbance at 280 nm and  
30 collected either manually or with a fraction collector (BioRad). Protein was found to elute at 200 and 400 mM imidazole only.

Figure 36 shows a Coomassie stained SDS-PAGE gel containing protein eluted during the imidazole step gradient. Lane 1 contains broad range MW markers (BioRad). Lane 2

contains BotB protein purified by IDA chromatography of an extract of pHisBotB/BL21(DE3) pLysS cells grown in shaker flask culture (*i.e.*, no co-expression of chaperones; Ex. 35).

Lane 3 contains a 20% w/v PEI clarified lysate of pHisBotB kan lacIq

T7/pACYCGro/BL21(DE3) cells (*i.e.*, the lysate prior to purification by IDA

5 chromatography). Lanes 4 and 5 contain protein which eluted at 200 or 400 mM imidazole, respectively. Lane 6 is blank. Lanes 7 and 8 contain 1/5 the load present in lanes 4 and 5.

As shown in Figure 36, both the chaperone and the BotB protein eluted in 200 mM imidazole, and more chaperone elutes in 400 mM imidazole, however no concentration of imidazole tested permitted the elution of BotB protein alone. Consequently, no significant  
10 purification was achieved using imidazole at these concentrations.

Because of the considerable difference in molecular weights between the folding chaperone, which is a multimer with a total molecular weight around 400 kD (as determined on a Shodex KB 804 sizing column by HPLC), and the recombinant BotB protein (molecular weight around 50 kD), size exclusion chromatography was next examined for the ability to  
15 separate these proteins.

#### a) Size Exclusion Chromatography

A column containing Sephacryl S-100 HR (S-100) (Pharmacia) was poured (2.5 cm x 24 cm : ~110 ml bed volume). The column was equilibrated in a buffer consisting of  
20 phosphate buffered saline (10mM potassium phosphate, 150 mM NaCl, pH 7.2) and 10 % glycerol (Mallinkrodt). Typically, 5 ml of the IDA-purified BotB protein was filtered through a 0.45  $\mu$  syringe filter and applied to the column, and the equilibration buffer was pumped through the column at a flow rate of 1 ml/minute. Eluted proteins were monitored by absorbance at 280 nm and collected either manually or with a fraction collector. Appropriate  
25 tubes were pooled, if necessary, and the protein was quantitated by absorbance at 280 nm and/or by BCA protein assay. The isolated peaks were then analyzed by native and/or SDS-PAGE to identify the protein and evaluate the purity.

Because of its larger size, the folding chaperone eluted first, followed by the recombinant BotB protein. A smaller third peak was observed which failed to stain when  
30 analyzed by SDS-PAGE and therefore was presumed to be imidazole.

SDS-PAGE analysis (12.5% polyacrylamide, reduced samples) was used to evaluate the purity of the IDA-purified recombinant BotB protein before and after S-100 purification. The results are shown in Figure 33.

In Figure 33, lane 1 contains broad range MW markers (BioRad). Lane 5 contains IDA-purified BotB protein. Lane 6 contains IDA-purified BotB protein following S-100 purification. Lane 7 is blank (lanes 2-4 were discussed in Ex. 34 above).

The results shown in Figure 33 show that the IDA-purified BotB is significantly contaminated with the folding chaperone (molecular weight about 60 kD under reducing conditions; lane 6). Following S-100 purification, the amount of folding chaperone present in the BotB sample was reduced dramatically (lane 7). Visual inspection of the Coomassie stained SDS-PAGE gel revealed that after S-100 purification, > 90% of the total protein present was BotB.

The IDA-purified BotB and the S-100-purified BotB samples were analyzed by HPLC on a size exclusion column (Shodex KB 804); this analysis revealed that the BotB protein represented 64% of the total protein in the IDA-purified sample and that following S-100 purification, the BotB protein represented >95% of the total protein in the sample.

The IDA-purified BotB material was also applied to a ACA 44 (SpectraPor, Houston, TX) column. The ACA 44 resin is equivalent to the S-100 resin and chromatography using the ACA 44 resin was carried out exactly as described above for the S-100 resin. The ACA 44 resin was found to separate the recombinant BotB protein from the folding chaperone. The ACA 44-purified BotB sample was analyzed for endotoxin using the LAL assay (Associates of Cape Cod) as describe in Example 24. Two aliquots of the ACA 44-purified BotB preparation were analyzed and were found to contain either 58 to 116 EU/mg recombinant protein or 94 to 189 EU/mg recombinant protein.

These results demonstrate that size exclusion chromatography can be used to purify the recombinant BotB protein from the folding chaperone and imidazole in IDA-purified material.

#### **b) Ultrafiltration For The Separation Of Recombinant BotB Protein And Chaperones**

Ultrafiltration was examined as an alternative method for the separation recombinant BotB protein and folding chaperones in IDA-purified material. While in this example only mixtures of BotB and chaperones were separated by ultrafiltration, this technique is suitable for use with recombinant BotA and BotE proteins as well provided that the wash buffers used are altered as necessary to take into account different requirements for solubility.

The recombinant BotB protein and folding chaperones were separated using a two-step sequential ultrafiltration method. The first membrane used had a nominal molecular weight

cutoff (MWCO) of approximately 100 kD; this membrane retains the larger folding chaperone while allowing the smaller recombinant protein to pass through. The addition of several volumes of wash buffer may be required to efficiently wash the recombinant protein through the membrane. The second step utilized a membrane with a nominal MWCO of approximately 10 kD. During this step, the recombinant antigen was retained by the membrane and could be concentrated to the degree desired and the imidazole and excess wash buffer passed through the membrane.

Twenty-seven milliliters of an IDA-purified BotB preparation was ultrafiltered through a 47 mm YM 100 (100 kD MWCO) membrane (Amicon) in a 50 ml stirred cell (Amicon). The membrane was washed in dd H<sub>2</sub>O prior to use as recommended by the manufacturer. Six volumes of 10% glycerol in PBS were washed through to remove most of the recombinant BotB protein and this wash was collected in a separate vessel. The resulting BotB protein-rich filtrate was then concentrated 12-fold using a YM 10 (10 kD MWCO) membrane (Amicon), to a final volume of 14 ml. The YM 100 and YM 10 concentrates were analyzed along with the lysate starting material by native PAGE using a 4 -15% pre-cast gradient gel (BioRad). The results are shown in Figure 37.

In Figure 37, lane 1 contains IDA-purified BotB derived from a shaker flask culture (*i.e.*, no co-expression of chaperones; Ex. 35); lane 2 contains a 20% w/v PEI clarified lysate of pHisBotB kan lacIq T7/pACYCGro/BL21(DE3) cells; lane 3 shows the lysate of lane 3 after IDA purification; lane 4 contains the YM 10 concentrate and lane 5 contains the YM 100 concentrate.

The results shown in Figure 37 demonstrate that the recombinant BotB protein can be purified away from the folding chaperone by ultrafiltration through a 100 kD MWCO membrane (lane 4), leaving the chaperone protein in the 100 kD concentrate (lane 5). Analysis of the sample in lane 5 also showed that very little of the BotB protein was retained by the 100 kD MWCO membrane after 6 volumes of wash buffer had been applied.

The BotB samples following IDA chromatography and following ultrafiltration through the YM 100 membrane were analyzed by HPLC on a size exclusion column (Shodex KB 804); this analysis revealed that the BotB protein represented 64% of the total protein in the IDA-purified sample and that following ultrafiltration through the YM 100 membrane, the BotB protein represented >96% of the total protein in the sample.

The BotB protein purified by ultrafiltration through the YM 100 membrane was examined for endotoxin using the LAL assay (Associates of Cape Cod) as describe in

Example 24. Two aliquots of the YM 100-purified BotB preparation were analyzed and were found to contain either 18 to 36 EU/mg recombinant protein or 125 to 250 EU/mg recombinant protein.

The above results demonstrate that size exclusion chromatography and ultrafiltration can be used to purify recombinant botulinum toxin proteins away from folding chaperones.

#### EXAMPLE 41

##### Cloning And Expression Of The C Fragment Of The *C. botulinum* Serotype E Toxin Gene

The *C. botulinum* type E neurotoxin gene has been cloned and sequenced from several different strains [Poulet *et al.* (1992) Biochem. Biophys. Res. Commun. 183:107 (strain Beluga); Whelan *et al.* (1992) Eur. J. Biochem. 204:657 (strain NCTC 11219); Fujii *et al.* (1990) Microbiol. Immunol. 34:1041 (partial sequence of strains Mashike, Iwani and Otaru) and Fujii *et al.* (1993) J. Gen. Microbiol. 139:79 (strain Mashike)]. The nucleotide sequence of the type E toxin gene is available from the EMBL sequence data bank under accession numbers X62089 (strain Beluga) and X62683 (strain NCTC 11219). The nucleotide sequence of the coding region (strain Beluga) is listed in SEQ ID NO:49. The amino acid sequence of the *C. botulinum* type E neurotoxin derived from strain Beluga is listed in SEQ ID NO:50. The nucleotide sequence of the coding region (strain NCTC 11219) is listed in SEQ ID NO:51. The amino acid sequence of the *C. botulinum* type E neurotoxin derived from strain NCTC 11219 is listed in SEQ ID NO:52.

The DNA sequence encoding the native *C. botulinum* serotype E C fragment gene derived from the Beluga strain can be expressed as a histidine-tagged protein using the pETHisb vector: the resulting coding region is listed in SEQ ID NO:53 and the corresponding amino acid sequence is listed in SEQ ID NO:54. The DNA sequence encoding the C fragment of the native *C. botulinum* serotype E gene derived from the NCTC 11219 strain can be expressed as a histidine-tagged fusion protein using the pETHisb vector: the resulting coding region is listed in SEQ ID NO:55 and the corresponding amino acid sequence is listed in SEQ ID NO:56. The C fragment region from any strain of *C. botulinum* serotype E can be amplified and expressed using the approach illustrated below using the C fragment derived from *C. botulinum* type E 2231 strain (ATCC #17786).



The type E neurotoxin gene is synthesized as a single polypeptide chain which may be converted to a double-chain form (*i.e.*, a heavy chain and a light chain) by cleavage with trypsin; unlike the type A neurotoxin, the type E neurotoxin exists essentially only in the single-chain form. The 50 kD carboxy-terminal portion of the heavy chain is referred to as the C fragment or the H<sub>c</sub> domain. Expression of the C fragment of *C. botulinum* type E toxin in heterologous hosts (*e.g.*, *E. coli*) has not been previously reported.

The native C fragment of the *C. botulinum* serotype E toxin (BotE) gene was cloned and inserted into expression vectors to facilitate expression of the recombinant BotE protein in *E. coli*. This example involved PCR amplification of the gene, cloning, and construction of expression vectors.

The BotE serotype gene was isolated using PCR as described for the BotA serotype gene in Example 28. The *C. botulinum* type E strain was obtained from the American Type Culture Collection (ATCC #17786; strain 2231). The following primer pair was used in the PCR amplification: 5'-CGCCATGGCTCTTTCTTCTTAT ACAGATGAT-3' (5' primer, engineered *Nco*I site underlined) (SEQ ID NO:57) and 5'-GCAAGCTT~~TTT~~ATTTTTCTTGCCATCCATG-3' (3' primer, engineered *Hind*III site underlined, native gene termination codon italicized) (SEQ ID NO:58). The PCR product was inserted into pCRscript as described in Example 28. The resulting pCRscript BotE clone was confirmed by restriction digestion, as well as, by obtaining the sequence of approximately 300 bases located at the 5' end of the C fragment coding region using standard DNA sequencing methods. The resulting BotE sequence was identical to that of the published *C. botulinum* type E toxin sequence [Whelan *et al* (1992), *supra*].

The *Nhe*I(filled)/*Hind*III fragment from a pCRscript BotE recombinant was cloned into pETHisb vector as described for BotA C fragment in Example 28. The resulting construct was termed pHisBotE. pHisBotE expresses the BotE gene under the control of the T7 lac promoter and the resulting protein contains an N-terminal 10XHis-tag affinity tag.

The pHisBotE expression construct was transformed into BL21(DE3) pLysS competent cells and 1 liter cultures were grown, induced and his-tagged proteins were purified utilizing a NiNTA resin (eluted in low pH elution buffer) as described in Example 28. Total, soluble and purified proteins were resolved by SDS-PAGE and detected by Coomassie staining. The results are shown in Figure 38.

In Figure 38, lane 1 contains broad range MW markers (BioRad); lane 2 contains a total protein extract; lane 3 contains a soluble protein extract; lane 4 contains proteins present

in the flow through from the NiNTA column (this sample was not diluted prior to loading and therefore represents a load 5X that of the load applied for the total and soluble extracts in lanes 2 and 3); lane 5 contains proteins eluted from the NiNTA column; lane 6 contains protein eluted from a NiNTA column which had been stored at -20°C for 1 year.

5 The pHisBotE protein was expressed at moderate levels (7 mg/liter) as a totally soluble protein. The purified protein migrated as a single band of the predicted MW.

Western blot hybridization utilizing a chicken anti-*C. botulinum* serotype E toxoid primary antibody (generated by immunization of hens as described in Example 3 using *C. botulinum* serotype E toxoid ) was also performed on the total, soluble and purified BotE proteins. Samples of BotA and BotB C fragments were also included on the gels to facilitate 10 MW and immunogenicity comparisons. Strong immunoreactivity was detected using the anti-*C. botulinum* type E toxoid antibody only with the BotE protein.

These results demonstrate that the native BotE gene sequences can be expressed as a soluble his-tagged protein in *E. coli* and purified by metal-chelation affinity chromatography.

#### EXAMPLE 42

##### Generation Of Neutralizing Antibodies Using The Recombinant pHisBotE Protein

20 The ability of the purified pHisBotE protein to generate neutralizing antibodies was examined. Nine BALBc mice were immunized with BotE protein (purified as described in Ex. 41) using Gerbu GMDP adjuvant (CC Biotech). The low pH elution was mixed with Gerbu adjuvant and used to immunize mice. Each mouse received a subcutaneous injection of 100 µl antigen/adjuvant mix (35 µg antigen + 1 µg adjuvant) on day 0. Mice were subcutaneously boosted as above on day 14 and bled on day 28. Mice were subsequently 25 boosted and bled on day 70.

Anti-*C. botulinum* serotype E toxoid titers were determined in day 28 serum from individual mice from each group using the ELISA protocol outlined in Example 29 with the exception that the plates were coated with *C. botulinum* serotype E toxoid, and the primary antibody was a chicken anti-*C. botulinum* serotype E toxoid. Seroconversion [relative to 30 control mice immunized with the p6xHisBotA antigen (Ex. 29)] was observed with all 9 mice immunized with the purified pHisBotE protein.

The ability of the anti-BotE antibodies to neutralize native *C. botulinum* type E toxin was tested in a mouse-*C. botulinum* neutralization model using pooled mouse serum (see Ex.

23b). The LD<sub>50</sub> of purified *C. botulinum* type E toxin complex (Dr. Eric Johnson, University of Wisconsin, Madison) was determined by a intraperitoneal (IP) method [Schantz and Kautler (1978), supra] using 18-22 g female ICR mice. The amount of neutralizing antibodies present in the serum of the immunized mice was determined using serum antibody titrations. The various serum dilutions (0.01 ml) were mixed with 5 LD<sub>50</sub> units of *C. botulinum* type E toxin and the mixtures were injected IP into mice. The neutralizations were performed in duplicate. The mice were then observed for signs of botulism for 4 days. Undiluted serum from day 28 did not protect, while undiluted, 1/10 diluted and 1/100 diluted day 70 serum protected (1005 of animals) while 1/1000 diluted day 70 serum did not. This corresponds to a neutralization titer of 50-500 IU/ml.

These results demonstrate that seroconversion occurred and neutralizing antibodies were induced when the recombinant BotE protein was utilized as the immunogen.

### EXAMPLE 43

#### Construction Of Vectors To Facilitate Expression Of His-Tagged BotE Protein In Fermentation Cultures

A number of expression vectors were constructed to facilitate the expression of recombinant BotE protein in large scale fermentation culture. These constructs varied as to the strength of the promoter utilized (T7 or T7lac) and the presence of repressor elements (lacIq) on the plasmid. The resulting constructs varied in the level of expression achieved and in plasmid stability which facilitated the selection of a optimal expression system for fermentation scaleup. This example involved a) construction of BotE expression vectors and b) determination of expression levels in small scale cultures.

##### a) Construction Of BotE Expression Vectors

The BotE expression vectors created for fermentation culture were engineered to utilize the kanamycin rather than the ampicillin resistance gene, and contained either the T7 or T7lac promoter, with or without the lacIq gene for the reasons outlined in Example 30.

In all cases, the protein expressed by the various expression vectors is the pHisBotE protein described in Example 41, with the only differences between clones being the alteration of various regulatory elements. Using the designations outlined below, the pHisBotE clone (Ex. 41) is equivalent to pHisBotE amp T7lac.

**i) Construction Of pHisBotE kan lacIq T7lac**

pHisBotE kan lacIq T7lac was constructed by inserting the *Xba*I/*Hind*III fragment of pHisBotE which contains the BotE gene sequences into *Xba*I/*Hind*III-cleaved pET24a vector. Proper construction was confirmed by restriction digestion.

**ii) Construction Of pHisBotE kan T7**

pHisBotE kan T7 was constructed by ligating the BotE-containing *Xba*I/*Sap*I fragment of pHisBotE kan lacIqT7lac to the T7 promoter-containing *Xba*I/*Sap*I fragment of pET23a. Proper construction was confirmed by restriction digestion.

**iii) Construction Of pHisBotE kan lacIqT7**

pHisBotE kan lacIqT7 was constructed by inserting the *Bgl*II/*Hind*III fragment from pHisBotE kan T7 which contains the BotE gene sequences into *Bgl*II/*Hind*III-cleaved pET24 vector. Proper construction was confirmed by restriction digestion.

**b) Determination Of BotE Expression Levels In Small Scale Cultures**

The three BotE kan expression vectors described above were transformed into B121(DE3) competent cells and 50 ml (2XYT + 40 µg/ml kan) cultures were grown and induced with IPTG as described in Example 28. Total and soluble protein extracts from before and after induction made as described in Example 28. The total and soluble extracts were resolved on a 12.5% SDS-PAGE gel, and his-tagged proteins were detected on a Western blot utilizing the NiNTA-alkaline phosphatase conjugate as described in Example 31(c)(iii). The results showed that all three BotE cell lines expressed his-tagged proteins of the predicted MW for the BotE protein upon induction. The results also demonstrated that the two constructs that contained the T7 promoter expressed the BotE protein before induction, while the T7lac promoter construct did not. Upon induction, the T7 promoter-containing constructs induced to higher levels than the T7lac-containing construct, with the pHisBotE kan lacIqT7/B121(DE3) cells accumulating the maximal levels of BotE protein.

**EXAMPLE 44****Expression And Purification Of pHisBotE From Fermentation Cultures**

Based on the small scale inductions performed in Example 43, the pHisBotE kan lacIq T7/B121(DE3) strain was selected for fermentation scaleup. This example involved the fermentation and purification of recombinant BotE C fragment protein.

A fermentation with the pHisBotE kan lacIq T7/B121(DE3) strain was performed as described in Example 31. The fermentation culture was induced 2 hrs post start of the glucose feed with 4 gm IPTG (final concentration = 1.6 mM). The OD<sub>600</sub> was 42 at time of induction, then 46.5, 48, 53 and 54 at 1-4 hrs post induction. Viable colony counts decreased from 0-4 hr induction [131, 4 (28), 7 (3), 7, 8; dilution 3 utilized 6 µl of dilution 2 cells; bracketed colonies are microcolonies]. All (32/32) colonies scored at the time of induction retained the BotE plasmid (kan resistant) and no colonies at induction grew on IPTG+Kan plates (no mutations detected). These results were indicative of strong promoter induction, since colony viability reduced after induction, and the culture stopped growing during fermentation (stopped at 54 OD<sub>600</sub>/ml).

Total and soluble extracts were resolved on a 12.5% SDS-PAGE gel and total protein was detected by staining with Coomassie blue. The results are shown in Figure 39.

In Figure 39, lane 1 contains total protein from a pHisBotA kan T7 lac/B121(DE3) pLysS fermentation (Ex. 24). Lanes 2-9 contain extracts prepared from the above pHisBotE kan lacIq T7/B121(DE3) fermentation: lanes 2- 4 contain total protein extracts prepared at 0, 1 and 2 hours post-induction, respectively. Lane 5 contains a soluble protein extract prepared at 2 hours post-induction. Lanes 6 and 7 contain total and soluble extracts prepared at 3 hours post-induction, respectively. Lanes 8 and 9 contain total and soluble extracts prepared at 4 hours post-induction, respectively. Lane 10 contains broad range MW markers (BioRad).

The results shown in Figure 39 demonstrate that moderate level induction of totally soluble Bot E protein was observed, increasing from 1 to 4 hrs post induction (no expression was detected in uninduced cells). From a 2 liter fermentation harvest a 155 gm (wet wt) cell pellet was obtained and used to make a PEI-clarified lysate (1 liter in CRB, pH 6.8). The lysate was applied to a large scale IDA column and 200 mg of BotE protein, which was found to be greater than 95% pure (as judged by visual inspection of a Coomassie stained SDS-PAGE gel), was recovered. This represents 2.5% of the total soluble cellular protein

(assuming a PEI lysate having a concentration of 8 mg protein/ml) and corresponds to a yield of 100 mg BotE protein/liter of fermentation culture.

The above results demonstrate that high levels of the recombinant BotE protein can be expressed and purified from fermentation cultures.

5

#### EXAMPLE 45

##### Removal Of Imidazole From Purified Recombinant BotE Protein Preparations

The expression of recombinant BotE protein, unlike the BotA and BotB proteins, did not require the presence of folding chaperones to maintain solubility during scale-up. A size exclusion chromatography step was included however to remove the imidazole from the sample and exchange the IDA elution buffer for one consistent with the BotA antigen.

A Sephacryl S-100 HR (S-100; Pharmacia) column was poured (2.5 cm x 24 cm; bed volume ~ 110 ml). Under these conditions, the BotE protein should be retained by the beads to a lesser degree than the smaller imidazole, therefore the BotE protein should elute from the column before the imidazole. The column was equilibrated in a buffer consisting of 50 mM sodium phosphate, 0.5 M NaCl, and 10% glycerol (all reagents from Mallinkrodt). Five milliliters of the IDA-purified BotE protein (Ex. 44) was filtered through a 0.45  $\mu$  syringe filter and applied to the S-100 column, and equilibration buffer was pumped through the column at a flow rate of 1 ml/minute. Eluted proteins were monitored by absorbance at 280 nm, and collected either manually or with a fraction collector. Appropriate tubes were pooled if necessary, and the protein was quantitated by absorbance at 280 nm and/or BCA protein assay. The isolated peaks were then analyzed by native and/or SDS-PAGE to identify the protein(s) and evaluate the purity.

Figure 40 provides a representative chromatogram showing the purification of IDA-purified BotE on the S-100 column. Even though folding chaperones were not over-expressed with this antigen, a small amount of protein eluted at a time consistent with the folding chaperones expressed with BotA and BotB proteins (Gro) (see the first peak). The second peak in the chromatogram contained the BotE protein, and the third peak was presumably imidazole. This presumed imidazole peak was isolated in comparable levels in IDA-purified BotA and BotB protein preparations as well.

These results demonstrate that size exclusion chromatography can be used to remove imidazole and traces of contaminating high molecular weight proteins from IDA-purified BotE protein preparations.

5 The S-100-purified BotE protein was tested for endotoxin contamination using the LAL assay as described in Example 24. This preparation was found to contain 64 to 128 EU/mg recombinant protein and is therefore substantially free of endotoxin.

10 The S-100 purified BotE was mixed with purified preparations of BotA and BotB proteins and used to immunize mice: 5 µg of each Bot protein was used per immunization and alum was included as an adjuvant. After two immunizations with this trivalent vaccine, the immunized mice were challenged with *C. botulinum* toxin. The immunized mice contained neutralizing antibodies sufficient to neutralize between 100,000 to 1,000,000 LD<sub>50</sub> of either toxin A or toxin B and between 1,000 to 10,000 LD<sub>50</sub> of toxin E. The titer of neutralizing antibodies directed against toxin E would be expected to increase following subsequent boosts with the vaccine. These results demonstrate that a trivalent vaccine  
15 containing recombinant BotA, BotB and BotE proteins provokes neutralizing antibodies.

#### EXAMPLE 46

##### Expression Of The C Fragment Of The *C. botulinum*

##### Serotype C Toxin Gene And Generation Of Neutralizing Antibodies

20

The *C. botulinum* type C1 neurotoxin gene has been cloned and sequenced [Kimura *et al.* (1990) Biochem. Biophys. Res. Comm. 171:1304]. The nucleotide sequence of the toxin gene derived from the *C. botulinum* type C strain C-Stockholm is available from the EMBL/GenBank sequence data banks under the accession number D90210; the nucleotide  
25 sequence of the coding region is listed in SEQ ID NO:59. The amino acid sequence of the *C. botulinum* type C1 neurotoxin derived from this strain is listed in SEQ ID NO:60.

The DNA sequence encoding the native *C. botulinum* serotype C1 C fragment gene derived from the C-Stockholm strain can be expressed using the pETHisb vector; the resulting coding region is listed in SEQ ID NO:61 and the corresponding amino acid sequence is listed  
30 in SEQ ID NO:62. The C fragment region from any strain of *C. botulinum* serotype C can be amplified and expressed using the approach illustrated below using the C fragment derived from *C. botulinum* type C C-Stockholm strain. Expression of the C fragment of *C. botulinum* type C1 toxin in heterologous hosts (*e.g.*, *E. coli*) has not been previously reported.

The C fragment of the *C. botulinum* serotype C1 (BotC1) toxin gene is cloned using the protocols and conditions described in Example 28 for the isolation of the native BotA gene. A number of *C. botulinum* serotype C strains (expressing either or both C1 and C2 toxin) are available from the ATCC [e.g., 2220 (ATCC 17782), 2239 (ATCC 17783), 2223 (ATCC 17784; a type C- $\beta$  strain; C- $\beta$  strains produce C2 toxin), 662 (ATCC 17849; a type C- $\alpha$  strain; C- $\alpha$  strains produce mainly C1 toxin and a small amount of C2 toxin), 2021 (ATCC 17850; a type C- $\alpha$  strain) and VPI 3803 (ATCC 25766)]. Alternatively, other type C strains may be employed for the isolation of sequences encoding the C fragment of *C. botulinum* serotype C toxin.

The following primer pair is used to amplify the BotC gene: 5'-CGCCATGGC TTTATTAAAAGATATAATTAATG-3' [5' primer, engineered *Nco*I site underlined (SEQ ID NO:63)] and 5'-GCAAGCTTTTATTCACTTACAGGTAC AAAACC-3' [3' primer, engineered *Hind*III site underlined, native gene termination codon italicized (SEQ ID NO:64)]. Following PCR amplification, the PCR product is inserted into the pCRscript vector and then the 1.5 kb fragment is cloned into pETHisb vector as described for BotA C fragment gene in Example 28. The resulting construct is termed pHisBotC. Proper construction is confirmed by DNA sequencing of the BotC sequences contained within pHisBotC.

pHisBotC expresses the BotC gene sequences under the transcriptional control of the T7 lac promoter and the resulting protein contains an N-terminal 10XHis-tag affinity tag. The pHisBotC expression construct is transformed into BL21(DE3) pLysS competent cells and 1 liter cultures are grown, induced and his-tagged proteins are purified utilizing a NiNTA resin (eluted in 250 mM imidazole, 20% glycerol) as described in Example 28. Total, soluble and purified proteins are resolved by SDS-PAGE and detected by Coomassie staining and Western blot hybridization utilizing a Ni-NTA-alkaline phosphatase conjugate (Qiagen) which recognizes his-tagged proteins as described in Example 31(c)(iii). This analysis permits the determination of expression levels of the pHisBotC protein (i.e., number of mg/liter expressed as a soluble protein). The purified BotC protein will migrate as a single band of the predicted MW (i.e., ~50kD).

The level of expression of the pHisBotC protein may be modified (increased) by substitution of the T7 promoter for the T7lac promoter, or by inclusion of the lacIq gene on the expression plasmid, and plasmid expressed in BL21(DE3) cell lines in fermentation cultures as described in Example 30. If only very low levels (i.e., less than 0.5%) of soluble pHisBotC protein are expressed using the above expression systems, the pHisBotC construct



may be co-expressed with pACYCGro construct as described in Example 32. In this case, the recombinant BotC protein may co-purify with the folding chaperones. The contaminating chaperones may be removed as described in Example 34. Preparations of purified pHisBotC protein are tested for endotoxin contamination using the LAL assay as described in Example 24.

The purified pHisBotC protein is used to generate neutralizing antibodies. BALBc mice are immunized with the BotC protein using Gerbu GMDP adjuvant (CC Biotech) as described in Example 36. The ability of the anti-BotC antibodies to neutralize native *C. botulinum* type C toxin is demonstrated using the mouse-*C. botulinum* neutralization model described in Example 36.

#### EXAMPLE 47

##### Expression Of The C Fragment Of The *C. botulinum* Serotype D Toxin Gene And Generation Of Neutralizing Antibodies

The *C. botulinum* type D neurotoxin gene has been cloned and sequenced [Sunagawa et al. (1992) J. Vet. Med. Sci. 54:905 and Binz et al. (1990) Nucleic Acids Res. 18:5556]. The nucleotide sequence of the toxin gene derived from the CB16 strain is available from the EMBL/GenBank sequence data banks under the accession number S49407; the nucleotide sequence of the coding region is listed in SEQ ID NO:65. The amino acid sequence of the *C. botulinum* type D neurotoxin derived from the CB16 strain is listed in SEQ ID NO:66.

The DNA sequence encoding the native *C. botulinum* serotype D C fragment gene derived from a BotD expressing strain can be expressed using the pETHisb vector; the resulting coding region is listed in SEQ ID NO:67 and the corresponding amino acid sequence is listed in SEQ ID NO:68. The C fragment region from any strain of *C. botulinum* serotype D can be amplified and expressed using the approach illustrated below using the C fragment derived from *C. botulinum* type D CB16 strain. Expression of the C fragment of *C. botulinum* type D toxin in heterologous hosts (e.g., *E. coli*) has not been previously reported.

The C fragment of the *C. botulinum* serotype D (BotD) toxin gene is cloned using the protocols and conditions described in Example 28 for the isolation of the native BotA gene. A number of *C. botulinum* type D strains are available from the ATCC [e.g., ATCC 9633, 2023 (ATCC 17851), and VPI 5995 (ATCC 27517)].

The following primer pair is used to amplify the BotD gene: 5'-CGCCATGGC TTTATTAAAAGATATAATTAATG-3' [5' primer, engineered *Nco*I site underlined (SEQ ID NO:63)] and 5'-GCAAGCTTTTACTCTACCCATCCTGGATCCCT-3' [3' primer, engineered *Hind*III site underlined, native gene termination codon italicized (SEQ ID NO:69)].

Following PCR amplification, the PCR product is inserted into the pCRscript vector and then the 1.5 kb fragment is cloned into pETHisb vector as described for BotA C<sub>1</sub> fragment gene in Example 28. The resulting construct is termed pHisBotD.

pHisBotD expresses the BotD gene sequences under the transcriptional control of the T7 lac promoter and the resulting protein contains an N-terminal 10XHis-tag affinity tag. The pHisBotD expression construct is transformed into BL21(DE3) pLysS competent cells and 1 liter cultures are grown, induced and his-tagged proteins are purified utilizing a NiNTA resin as described in Example 28. Total, soluble and purified proteins are resolved by SDS-PAGE and detected by Coomassie staining and Western blot hybridization utilizing a Ni-NTA-alkaline phosphatase conjugate (Qiagen) which recognizes his-tagged proteins as described in Example 31(c)(iii). This analysis permits the determination of expression levels of the pHisBotD protein (*i.e.*, number of mg/liter expressed as a soluble protein). The purified BotD protein will migrate as a single band of the predicted MW (*i.e.*, ~50kD).

The level of expression of the pHisBotD protein may be modified (increased) by substitution of the T7 promoter for the T7lac promoter, or by inclusion of the lacI<sub>q</sub> gene on the expression plasmid, and plasmid expressed in BL21(DE3) cell lines in fermentation cultures as described in Example 30. If only very low levels (*i.e.*, less than about 0.5%) of soluble pHisBotD protein are expressed using the above expression systems, the pHisBotD construct may be co-expressed with pACYCGro construct as described in Example 32. In this case, the recombinant BotD protein may co-purify with the folding chaperones. The contaminating chaperones may be removed as described in Example 34. Preparations of purified pHisBotD protein are tested for endotoxin contamination using the LAL assay as described in Example 24.

The purified pHisBotD protein is used to generate neutralizing antibodies. BALBc mice are immunized with the BotD protein using Gerbu GMDP adjuvant (CC Biotech) as described in Example 36. The ability of the anti-BotD antibodies to neutralize native *C. botulinum* type D toxin is demonstrated using the mouse-*C. botulinum* neutralization model described in Example 36.

**EXAMPLE 48**

Expression Of The C Fragment Of The *C. botulinum*  
Serotype F Toxin Gene And Generation Of Neutralizing Antibodies

5       The *C. botulinum* type F neurotoxin gene has been cloned and sequenced [East *et al.*  
(1992) FEMS Microbiol. Lett. 96:225]. The nucleotide sequence of the toxin gene derived  
10       from the 202F strain (ATCC 23387) is available from the EMBL/GenBank sequence data  
banks under the accession number M92906; the nucleotide sequence of the coding region is  
listed in SEQ ID NO:70. The amino acid sequence of the *C. botulinum* type F neurotoxin  
derived from the 202F strain is listed in SEQ ID NO:71.

      The DNA sequence encoding the native *C. botulinum* serotype F C fragment gene  
derived from the 202F strain can be expressed using the pETHisb vector; the resulting coding  
region is listed in SEQ ID NO:72 and the corresponding amino acid sequence is listed in SEQ  
ID NO:73. The C fragment region from any strain of *C. botulinum* serotype F can be  
15       amplified and expressed using the approach illustrated below using the C fragment derived  
from *C. botulinum* type F 202F strain. Expression of the C fragment of *C. botulinum* type F  
toxin in heterologous hosts (*e.g.*, *E. coli*) has not been previously reported.

      The C fragment of the *C. botulinum* serotype F (BotF) toxin gene is cloned using the  
protocols and conditions described in Example 28 for the isolation of the native BotA gene.  
20       The *C. botulinum* type F 202F strain is obtained from the American Type Culture Collection  
(ATCC 23387). Alternatively, sequences encoding the BotF toxin may be isolated from any  
BotF expressing strain [*e.g.*, VPI 4404 (ATCC 25764), VPI 2382 (ATCC 27321) and  
Langeland (ATCC 35415)].

      The following primer pair is used to amplify the BotF gene: 5'-CGCCATGGC  
25       TATTCTAATTATATATTTTAATAG-3' [5' primer, engineered *Nco*I site underlined (SEQ  
ID NO:74)] and 5'-GCAAGCTTTCATTCTTTCCATCCATTCTC-3' [3' primer, engineered  
*Hind*III site underlined, native gene termination codon italicized (SEQ ID NO:75)].  
Following PCR amplification, the PCR product is inserted into the pCRscript vector and then  
the 1.5 kb fragment is cloned into pETHisb vector as described for BotA C fragment gene in  
30       Example 28. The resulting construct is termed pHisBotF.

      pHisBotF expresses the BotF gene sequences under the transcriptional control of the  
T7 lac promoter and the resulting protein contains an N-terminal 10XHis-tag affinity tag. The  
pHisBotF expression construct is transformed into BL21(DE3) pLysS competent cells and 1

liter cultures are grown, induced and his-tagged proteins are purified utilizing a NiNTA resin as described in Example 28. Total, soluble and purified proteins are resolved by SDS-PAGE and detected by Coomassie staining and Western blot hybridization utilizing a Ni-NTA-alkaline phosphatase conjugate (Qiagen) which recognizes his-tagged proteins as described in Example 31(c)(iii). This analysis permits the determination of expression levels of the pHisBotF protein (*i.e.*, number of mg/liter expressed as a soluble protein). The purified BotF protein will migrate as a single band of the predicted MW (*i.e.*, ~50kD).

The level of expression of the pHisBotF protein may be modified (increased) by substitution of the T7 promoter for the T7lac promoter, or by inclusion of the *lacIq* gene on the expression plasmid, and plasmid expressed in BL21(DE3) cell lines in fermentation cultures as described in Example 30. If only very low levels (*i.e.*, less than about 0.5%) of soluble pHisBotF protein are expressed using the above expression systems, the pHisBotF construct may be co-expressed with pACYCGro construct as described in Example 32. In this case, the recombinant BotF protein may co-purify with the folding chaperones. The contaminating chaperones may be removed as described in Example 34. Preparations of purified pHisBotF protein are tested for endotoxin contamination using the LAL assay as described in Example 24.

The purified pHisBotF protein is used to generate neutralizing antibodies. BALBc mice are immunized with the BotF protein using Gerbu GMDP adjuvant (CC Biotech) as described in Example 36. The ability of the anti-BotF antibodies to neutralize native *C. botulinum* type F toxin is demonstrated using the mouse-*C. botulinum* neutralization model described in Example 36.

#### EXAMPLE 49

##### Expression Of The C Fragment Of The *C. botulinum* Serotype G Toxin Gene And Generation Of Neutralizing Antibodies

The *C. botulinum* type G neurotoxin gene has been cloned and sequenced [Campbell *et al.* (1993) *Biochimica et Biophysica Acta* 1216:487 and Binz *et al.* (1990) *Nucleic Acids Res.* 18:5556]. The nucleotide sequence of the toxin gene derived from the 113/30 strain (NCFB 3012) is available from the EMBL/GenBank sequence data banks under the accession number X74162; the nucleotide sequence of the coding region is listed in SEQ ID NO:76. The amino

acid sequence of the *C. botulinum* type G neurotoxin derived from this strain is listed in SEQ ID NO:77.

The DNA sequence encoding the native *C. botulinum* serotype G C fragment gene derived from the 113/30 strain can be expressed using the pETHisb vector; the resulting coding region is listed in SEQ ID NO:78 and the corresponding amino acid sequence is listed in SEQ ID NO:79. The C fragment region from any strain of *C. botulinum* serotype G can be amplified and expressed using the approach illustrated below using the C fragment derived from *C. botulinum* type G 113/30 strain. Expression of the C fragment of *C. botulinum* type G toxin in heterologous hosts (e.g., *E. coli*) has not been previously reported.

The C fragment of the *C. botulinum* serotype G (BotG) toxin gene is cloned using the protocols and conditions described in Example 28 for the isolation of the native BotA gene. The *C. botulinum* type G 113/30 strain is obtained from the NCFB. The following primer pair is used to amplify the BotG gene: 5'-CGCCATGGCTGAC ACAATTTTAATACA AGT-3' [5' primer, engineered *NcoI* site underlined (SEQ ID NO:80)] and 5'-GCCTCGAGTTATTCTGTCCATCCTTCATCCAC-3' [3' primer, engineered *XhoI* site underlined, native gene termination codon italicized (SEQ ID NO:81)]. Following PCR amplification, the PCR product is inserted into the pCRscript vector and then the 1.5 kb fragment is cloned into pETHisb vector as described for BotA C fragment gene in Example 28 with the exception that the sequences encoding BotG are excised from the pCRscript vector by digestion with *NcoI* and *XhoI* and the *NcoI* site is blunted (the BotG sequences contain an internal *HindIII* site). This *NcoI*(filled)/*XhoI* fragment is then ligated to the pETHisb vector which has been digested with *NheI* and *Sall* and the *NheI* site is blunted. The resulting construct is termed pHisBotG.

pHisBotG expresses the BotG gene sequences under the transcriptional control of the T7 lac promoter and the resulting protein contains an N-terminal 10XHis-tag affinity tag. The pHisBotG expression construct is transformed into BL21(DE3) pLysS competent cells and 1 liter cultures are grown, induced and his-tagged proteins are purified utilizing a NiNTA resin as described in Example 28. Total, soluble and purified proteins are resolved by SDS-PAGE and detected by Coomassie staining and Western blot hybridization utilizing a Ni-NTA-alkaline phosphatase conjugate (Qiagen) which recognizes his-tagged proteins as described in Example 31(c)(iii). This analysis permits the determination of expression levels of the pHisBotG protein (i.e., number of mg/liter expressed as a soluble protein). The purified BotG protein will migrate as a single band of the predicted MW (i.e., ~50kD).

The level of expression of the pHisBotG protein may be modified (increased) by substitution of the T7 promoter for the T7lac promoter, or by inclusion of the lacIq gene on the expression plasmid, and plasmid expressed in BL21(DE3) cell lines in fermentation cultures as described in Example 30. If only very low levels (*i.e.*, less than about 0.5%) of soluble pHisBotG protein are expressed using the above expression systems, the pHisBotG construct may be co-expressed with pACYCGro construct as described in Example 32. In this case, the recombinant BotG protein may co-purify with the folding chaperones. The contaminating chaperones may be removed as described in Example 34. Preparations of purified pHisBotG protein are tested for endotoxin contamination using the LAL assay as described in Example 24.

The purified pHisBotG protein is used to generate neutralizing antibodies. BALBc mice are immunized with the BotG protein using Gerbu GMDP adjuvant (CC Biotech) as described in Example 36. The ability of the anti-BotG antibodies to neutralize native *C. botulinum* type G toxin is demonstrated using the mouse-*C. botulinum* neutralization model described in Example 36.

#### EXAMPLE 50

##### Expression Of Recombinant Botulinal Toxin Proteins In Eucaryotic Host Cells

Recombinant botulinal C fragment proteins may be expressed in eucaryotic host cells, such as yeast and insect cells.

##### a) Expression In Yeast

Botulinal C fragments derived from serotypes A, B, C, D, E, F and G may be expressed in yeast cells using a variety of commercially available vectors. For example, the pPIC3K and pPIC9K expression vectors (Invitrogen) may be employed for expression in the methylotrophic yeast, *Pichia pastoris*. When the pPIC3K vector is employed, expression of the botulinal C fragment protein will be intracellular. When the pPIC9K vector is employed, the botulinal C fragment protein will be secreted (the alpha factor secretion signal is provided on the pPIC9K vector).

DNA sequences encoding the desired C fragment is inserted into these vectors using techniques known to the art. Briefly, the desired botulinal expression cassette (including sequences encoding the his-tag, described in the preceding examples) is amplified using the

PCR in conjunction with primers that incorporate unique restriction sites at the termini of the amplified fragment. Suitable restriction enzyme sites include *Sna*BI, *Eco*RI, *Avr*II and *Not*I. When the botulinal C fragment is to be expressed using the pPIC3K vector, the initiator methionine (ATG) is provided by the desired Bot gene sequence and a Kozak consensus sequence is engineered upstream of the ATG (*e.g.*, ACCATGG).

The amplified restriction fragment containing the botulinal C fragment gene is then cloned into the desired expression vector. Recombinant clones are integrated into the *Pichia pastoris* genome and recombinant protein expression is induced using methanol following the manufacturer's instructions (Invitrogen Pichia expression kit manual).

*C. botulinum* genes are A/T rich and contain multiple sequences that are similar to yeast transcriptional termination signals (*e.g.*, TTTTATA). If premature transcription termination is observed when the botulinal C fragment genes are expressed in yeast, the transcription termination signals present in the C fragment genes can be removed by either site directed mutagenesis (utilizing the pALTER system; Promega) or by construction of synthetic genes utilizing overlapping synthetic primers.

The botulinal C fragment genes may be expressed in other yeast cells using other commercially available vectors [*e.g.*, using the pYES2 vector (Invitrogen) and *S. cerevisiae* cells (Invitrogen)].

#### **b) Expression In Insect Cells**

Botulinal C fragments derived from serotypes A, B, C, D, E, F and G may be expressed in insect cells using a variety of commercially available vectors. For example, the pBlueBac4 transfer vector (Invitrogen) may be employed for expression in *Spodoptera frugiperda* (Sf9) insect cells (baculovirus expression system) (equivalent baculovirus vectors and host cells are available from other vendors, *e.g.*, Pharmingen, San Diego, CA). Botulinal C fragments contained on *Nco*I/*Hind*III fragments contained within the pHisBotA-G expression constructs (described in the preceding examples) are cloned into the pBlueBac4 vector (digested with *Nco*I and *Hind*III); the *Nco*I site present on the C fragment constructs overlaps with the start codon of the fusion proteins. In the case of botulinal C fragment clones that contain internal *Hind*III sites (*e.g.*, using the BotG sequences described in Ex. 49), the C fragment gene is contained within a *Nco*I/*Xho*I fragment on the pHisBot construct. This *Nco*I/*Xho*I fragment is excised from pHisBot and inserted into pBlueBac4 digested with *Nco*I and *Sal*I. Recombinant baculoviruses are made and the desired recombinant C fragment

is expressed in *S*/9 cells using the protocols provided by the manufacturer (Invitrogen MaxBac manual). The resulting constructs will express the pHisBot protein intracellularly (including the N-terminal his-tag) under the control of the polyhedrin promoter. For extracellular secretion of botulinal C fragment proteins, the C fragment sequences from the pHisBot constructs are cloned into the pMelBacB vector (Invitrogen) as described above for the pBlueBac4 vector. When the pMelBacB vector is employed, the his-tagged botulinal C fragment proteins are secreted (utilizing a vector-encoded honeybee melittin secretion signal) and contain a nine amino acid extension at the N-terminus.

His-tagged botulinal C fragments expressed in yeast or insect cells are purified using metal chelation columns as described in the preceding examples.

From the above it is clear that the present invention provides compositions and methods for the preparation of effective multivalent vaccines against *C. botulinum* neurotoxin. It is also contemplated that the recombinant botulinal proteins be used for the production of antitoxins. All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described method and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention.



## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- 5 (i) APPLICANT: Williams, James A.  
Thalley, Bruce S.
- 10 (ii) TITLE OF INVENTION: Multivalent Vaccine For Clostridium  
Botulinum Neurotoxin
- (iii) NUMBER OF SEQUENCES: 82
- 15 (iv) CORRESPONDENCE ADDRESS:  
(A) ADDRESSEE: Medlen & Carroll  
(B) STREET: 220 Montgomery Street, Suite 2200  
(C) CITY: San Francisco  
(D) STATE: California  
(E) COUNTRY: United States of America  
20 (F) ZIP: 94104
- (v) COMPUTER READABLE FORM:  
(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
25 (C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:  
(A) APPLICATION NUMBER: US  
30 (B) FILING DATE:  
(C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:  
(A) NAME: Carroll, Peter G.  
35 (B) REGISTRATION NUMBER: 32,837  
(C) REFERENCE/DOCKET NUMBER: OPHD-02959
- (ix) TELECOMMUNICATION INFORMATION:  
(A) TELEPHONE: (415) 705-8410  
40 (B) TELEFAX: (415) 397-8338

## (2) INFORMATION FOR SEQ ID NO:1:

- 45 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 24 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- 50 (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GGAAATTTAG CTGCAGCATC TGAC

24

## 55 (2) INFORMATION FOR SEQ ID NO:2:

- 60 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 24 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)

## 65 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TCTAGCAAAT TCGCTTGTGT TGAA

24

## 70 (2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 20 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:  
 CTCGCATATA GCATTAGACC

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 19 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:  
 CTATCTAGGC CTAAAGTAT

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 8133 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:  
 (A) NAME/KEY: CDS  
 (B) LOCATION: 1..8130

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

ATG TCT TTA ATA TCT AAA GAA GAG TTA ATA AAA CTC GCA TAT AGC ATT	48
Met Ser Leu Ile Ser Lys Glu Glu Leu Ile Lys Leu Ala Tyr Ser Ile	
1 5 10 15	
AGA CCA AGA GAA AAT GAG TAT AAA ACT ATA CTA ACT AAT TTA GAC GAA	96
Arg Pro Arg Glu Asn Glu Tyr Lys Thr Ile Leu Thr Asn Leu Asp Glu	
20 25 30	
TAT AAT AAG TTA ACT ACA AAC AAT AAT GAA AAT AAA TAT TTG CAA TTA	144
Tyr Asn Lys Leu Thr Thr Asn Asn Asn Glu Asn Lys Tyr Leu Gln Leu	
35 40 45	
AAA AAA CTA AAT GAA TCA ATT GAT GTT TTT ATG AAT AAA TAT AAA ACT	192
Lys Lys Leu Asn Glu Ser Ile Asp Val Phe Met Asn Lys Tyr Lys Thr	
50 55 60	
TCA AGC AGA AAT AGA GCA CTC TCT AAT CTA AAA AAA GAT ATA TTA AAA	240
Ser Ser Arg Asn Arg Ala Leu Ser Asn Leu Lys Lys Asp Ile Leu Lys	
65 70 75 80	
GAA GTA ATT CTT ATT AAA AAT TCC AAT ACA AGC CCT GTA GAA AAA AAT	288
Glu Val Ile Leu Ile Lys Asn Ser Asn Thr Ser Pro Val Glu Lys Asn	
85 90 95	
TTA CAT TTT GTA TGG ATA GGT GGA GAA GTC AGT GAT ATT GCT CTT GAA	336
Leu His Phe Val Trp Ile Gly Gly Glu Val Ser Asp Ile Ala Leu Glu	
100 105 110	

	TAC ATA AAA CAA TGG GCT GAT ATT AAT GCA GAA TAT AAT ATT AAA CTG Tyr Ile Lys Gln Trp Ala Asp Ile Asn Ala Glu Tyr Asn Ile Lys Leu 115 120 125	384
5	TGG TAT GAT AGT GAA GCA TTC TTA GTA AAT ACA CTA AAA AAG GCT ATA Trp Tyr Asp Ser Glu Ala Phe Leu Val Asn Thr Leu Lys Lys Ala Ile 130 135 140	432
10	GTT GAA TCT TCT ACC ACT GAA GCA TTA CAG CTA CTA GAG GAA GAG ATT Val Glu Ser Ser Thr Thr Glu Ala Leu Gln Leu Leu Glu Glu Glu Ile 145 150 155 160	480
15	CAA AAT CCT CAA TTT GAT AAT ATG AAA TTT TAC AAA AAA AGG ATG GAA Gln Asn Pro Gln Phe Asp Asn Met Lys Phe Tyr Lys Lys Arg Met Glu 165 170 175	528
20	TTT ATA TAT GAT AGA CAA AAA AGG TTT ATA AAT TAT TAT AAA TCT CAA Phe Ile Tyr Asp Arg Gln Lys Arg Phe Ile Asn Tyr Tyr Lys Ser Gln 180 185 190	576
25	ATC AAT AAA CCT ACA GTA CCT ACA ATA GAT GAT ATT ATA AAG TCT CAT Ile Asn Lys Pro Thr Val Pro Thr Ile Asp Asp Ile Ile Lys Ser His 195 200 205	624
30	CTA GTA TCT GAA TAT AAT AGA GAT GAA ACT GTA TTA GAA TCA TAT AGA Leu Val Ser Glu Tyr Asn Arg Asp Glu Thr Val Leu Glu Ser Tyr Arg 210 215 220	672
35	ACA AAT TCT TTG AGA AAA ATA AAT AGT AAT CAT GGG ATA GAT ATC AGG Thr Asn Ser Leu Arg Lys Ile Asn Ser Asn His Gly Ile Asp Ile Arg 225 230 235 240	720
40	GCT AAT AGT TTG TTT ACA GAA CAA GAG TTA TTA AAT ATT TAT AGT CAG Ala Asn Ser Leu Phe Thr Glu Gln Glu Leu Leu Asn Ile Tyr Ser Gln 245 250 255	768
45	GAG TTG TTA AAT CGT GGA AAT TTA GCT GCA GCA TCT GAC ATA GTA AGA Glu Leu Leu Asn Arg Gly Asn Leu Ala Ala Ala Ser Asp Ile Val Arg 260 265 270	816
50	TTA TTA GCC CTA AAA AAT TTT GGC GGA GTA TAT TTA GAT GTT GAT ATG Leu Leu Ala Leu Lys Asn Phe Gly Gly Val Tyr Leu Asp Val Asp Met 275 280 285	864
55	CTT CCA GGT ATT CAC TCT GAT TTA TTT AAA ACA ATA TCT AGA CCT AGC Leu Pro Gly Ile His Ser Asp Leu Phe Lys Thr Ile Ser Arg Pro Ser 290 295 300	912
60	TCT ATT GGA CTA GAC CGT TGG GAA ATG ATA AAA TTA GAG GCT ATT ATG Ser Ile Gly Leu Asp Arg Trp Glu Met Ile Lys Leu Glu Ala Ile Met 305 310 315 320	960
65	AAG TAT AAA AAA TAT ATA AAT AAT TAT ACA TCA GAA AAC TTT GAT AAA Lys Tyr Lys Lys Tyr Ile Asn Asn Tyr Thr Ser Glu Asn Phe Asp Lys 325 330 335	1008
70	CTT GAT CAA CAA TTA AAA GAT AAT TTT AAA CTC ATT ATA GAA AGT AAA Leu Asp Gln Gln Leu Lys Asp Asn Phe Lys Leu Ile Ile Glu Ser Lys 340 345 350	1056
75	AGT GAA AAA TCT GAG ATA TTT TCT AAA TTA GAA AAT TTA AAT GTA TCT Ser Glu Lys Ser Glu Ile Phe Ser Lys Leu Glu Asn Leu Asn Val Ser 355 360 365	1104
80	GAT CTT GAA ATT AAA ATA GCT TTC GCT TTA GGC AGT GTT ATA AAT CAA Asp Leu Glu Ile Lys Ile Ala Phe Ala Leu Gly Ser Val Ile Asn Gln 370 375 380	1152

	GCC TTG ATA TCA AAA CAA GGT TCA TAT CTT ACT AAC CTA GTA ATA GAA Ala Leu Ile Ser Lys Gln Gly Ser Tyr Leu Thr Asn Leu Val Ile Glu 385 390 395 400	1200
5	CAA GTA AAA AAT AGA TAT CAA TTT TTA AAC CAA CAC CTT AAC CCA GCC Gln Val Lys Asn Arg Tyr Gln Phe Leu Asn Gln His Leu Asn Pro Ala 405 410 415	1248
10	ATA GAG TCT GAT AAT AAC TTC ACA GAT ACT ACT AAA ATT TTT CAT GAT Ile Glu Ser Asp Asn Asn Phe Thr Asp Thr Thr Lys Ile Phe His Asp 420 425 430	1296
15	TCA TTA TTT AAT TCA GCT ACC GCA GAA AAC TCT ATG TTT TTA ACA AAA Ser Leu Phe Asn Ser Ala Thr Ala Glu Asn Ser Met Phe Leu Thr Lys 435 440 445	1344
20	ATA GCA CCA TAC TTA CAA GTA GGT TTT ATG CCA GAA GCT CGC TCC ACA Ile Ala Pro Tyr Leu Gln Val Gly Phe Met Pro Glu Ala Arg Ser Thr 450 455 460	1392
	ATA AGT TTA AGT GGT CCA GGA GCT TAT GCG TCA GCT TAC TAT GAT TTC Ile Ser Leu Ser Gly Pro Gly Ala Tyr Ala Ser Ala Tyr Tyr Asp Phe 465 470 475 480	1440
25	ATA AAT TTA CAA GAA AAT ACT ATA GAA AAA ACT TTA AAA GCA TCA GAT Ile Asn Leu Gln Glu Asn Thr Ile Glu Lys Thr Leu Lys Ala Ser Asp 485 490 495	1488
30	TTA ATA GAA TTT AAA TTC CCA GAA AAT AAT CTA TCT CAA TTG ACA GAA Leu Ile Glu Phe Lys Phe Pro Glu Asn Asn Leu Ser Gln Leu Thr Glu 500 505 510	1536
35	CAA GAA ATA AAT AGT CTA TGG AGC TTT GAT CAA GCA AGT GCA AAA TAT Gln Glu Ile Asn Ser Leu Trp Ser Phe Asp Gln Ala Ser Ala Lys Tyr 515 520 525	1584
40	CAA TTT GAG AAA TAT GTA AGA GAT TAT ACT GGT GGA TCT CTT TCT GAA Gln Phe Glu Lys Tyr Val Arg Asp Tyr Thr Gly Gly Ser Leu Ser Glu 530 535 540	1632
	GAC AAT GGG GTA GAC TTT AAT AAA AAT ACT GCC CTC GAC AAA AAC TAT Asp Asn Gly Val Asp Phe Asn Lys Asn Thr Ala Leu Asp Lys Asn Tyr 545 550 555 560	1680
45	TTA TTA AAT AAT AAA ATT CCA TCA AAC AAT GTA GAA GAA GCT GGA AGT Leu Leu Asn Asn Lys Ile Pro Ser Asn Asn Val Glu Glu Ala Gly Ser 565 570 575	1728
50	AAA AAT TAT GTT CAT TAT ATC ATA CAG TTA CAA GGA GAT GAT ATA AGT Lys Asn Tyr Val His Tyr Ile Ile Gln Leu Gln Gly Asp Asp Ile Ser 580 585 590	1776
55	TAT GAA GCA ACA TGC AAT TTA TTT TCT AAA AAT CCT AAA AAT AGT ATT Tyr Glu Ala Thr Cys Asn Leu Phe Ser Lys Asn Pro Lys Asn Ser Ile 595 600 605	1824
60	ATT ATA CAA CGA AAT ATG AAT GAA AGT GCA AAA AGC TAC TTT TTA AGT Ile Ile Gln Arg Asn Met Asn Glu Ser Ala Lys Ser Tyr Phe Leu Ser 610 615 620	1872
	GAT GAT GGA GAA TCT ATT TTA GAA TTA AAT AAA TAT AGG ATA CCT GAA Asp Asp Gly Glu Ser Ile Leu Glu Leu Asn Lys Tyr Arg Ile Pro Glu 625 630 635 640	1920
65	AGA TTA AAA AAT AAG GAA AAA GTA AAA GTA ACC TTT ATT GGA CAT GGT Arg Leu Lys Asn Lys Glu Lys Val Lys Val Thr Phe Ile Gly His Gly 645 650 655	1968

	AAA GAT GAA TTC AAC ACA AGC GAA TTT GCT AGA TTA AGT GTA GAT TCA Lys Asp Glu Phe Asn Thr Ser Glu Phe Ala Arg Leu Ser Val Asp Ser	2016
	660 665 670	
5	CTT TCC AAT GAG ATA AGT TCA TTT TTA GAT ACC ATA AAA TTA GAT ATA Leu Ser Asn Glu Ile Ser Ser Phe Leu Asp Thr Ile Lys Leu Asp Ile	2064
	675 680 685	
10	TCA CCT AAA AAT GTA GAA GTA AAC TTA CTT GGA TGT AAT ATG TTT AGT Ser Pro Lys Asn Val Glu Val Asn Leu Leu Gly Cys Asn Met Phe Ser	2112
	690 695 700	
15	TAT GAT TTT AAT GTT GAA GAA ACT TAT CCT GGG AAG TTG CTA TTA AGT Tyr Asp Phe Asn Val Glu Glu Thr Tyr Pro Gly Lys Leu Leu Leu Ser	2160
	705 710 715 720	
20	ATT ATG GAC AAA ATT ACT TCC ACT TTA CCT GAT GTA AAT AAA AAT TCT Ile Met Asp Lys Ile Thr Ser Thr Leu Pro Asp Val Asn Lys Asn Ser	2208
	725 730 735	
25	ATT ACT ATA GGA GCA AAT CAA TAT GAA GTA AGA ATT AAT AGT GAG GGA Ile Thr Ile Gly Ala Asn Gln Tyr Glu Val Arg Ile Asn Ser Glu Gly	2256
	740 745 750	
30	AGA AAA GAA CTT CTG GCT CAC TCA GGT AAA TGG ATA AAT AAA GAA GAA Arg Lys Glu Leu Leu Ala His Ser Gly Lys Trp Ile Asn Lys Glu Glu	2304
	755 760 765	
35	GCT ATT ATG AGC GAT TTA TCT AGT AAA GAA TAC ATT TTT TTT GAT TCT Ala Ile Met Ser Asp Leu Ser Ser Lys Glu Tyr Ile Phe Phe Asp Ser	2352
	770 775 780	
40	ATA GAT AAT AAG CTA AAA GCA AAG TCC AAG AAT ATT CCA GGA TTA GCA Ile Asp Asn Lys Leu Lys Ala Lys Ser Lys Asn Ile Pro Gly Leu Ala	2400
	785 790 795 800	
45	TCA ATA TCA GAA GAT ATA AAA ACA TTA TTA CTT GAT GCA AGT GTT AGT Ser Ile Ser Glu Asp Ile Lys Thr Leu Leu Leu Asp Ala Ser Val Ser	2448
	805 810 815	
50	CCT GAT ACA AAA TTT ATT TTA AAT AAT CTT AAG CTT AAT ATT GAA TCT Pro Asp Thr Lys Phe Ile Leu Asn Asn Leu Lys Leu Asn Ile Glu Ser	2496
	820 825 830	
55	TCT ATT GGG GAT TAC ATT TAT TAT GAA AAA TTA GAG CCT GTT AAA AAT Ser Ile Gly Asp Tyr Ile Tyr Tyr Glu Lys Leu Glu Pro Val Lys Asn	2544
	835 840 845	
60	ATA ATT CAC AAT TCT ATA GAT GAT TTA ATA GAT GAG TTC AAT CTA CTT Ile Ile His Asn Ser Ile Asp Asp Leu Ile Asp Glu Phe Asn Leu Leu	2592
	850 855 860	
65	GAA AAT GTA TCT GAT GAA TTA TAT GAA TTA AAA AAA TTA AAT AAT CTA Glu Asn Val Ser Asp Glu Leu Tyr Glu Leu Lys Lys Leu Asn Asn Leu	2640
	865 870 875 880	
70	GAT GAG AAG TAT TTA ATA TCT TTT GAA GAT ATC TCA AAA AAT AAT TCA Asp Glu Lys Tyr Leu Ile Ser Phe Glu Asp Ile Ser Lys Asn Asn Ser	2688
	885 890 895	
75	ACT TAC TCT GTA AGA TTT ATT AAC AAA AGT AAT GGT GAG TCA GTT TAT Thr Tyr Ser Val Arg Phe Ile Asn Lys Ser Asn Gly Glu Ser Val Tyr	2736
	900 905 910	
80	GTA GAA ACA GAA AAA GAA ATT TTT TCA AAA TAT AGC GAA CAT ATT ACA Val Glu Thr Glu Lys Glu Ile Phe Ser Lys Tyr Ser Glu His Ile Thr	2784
	915 920 925	

	AAA GAA ATA AGT ACT ATA AAG AAT AGT ATA ATT ACA GAT GTT AAT GGT Lys Glu Ile Ser Thr Ile Lys Asn Ser Ile Ile Thr Asp Val Asn Gly	2832
	930 935 940	
5	AAT TTA TTG GAT AAT ATA CAG TTA GAT CAT ACT TCT CAA GTT AAT ACA Asn Leu Leu Asp Asn Ile Gln Leu Asp His Thr Ser Gln Val Asn Thr	2880
	945 950 955 960	
10	TTA AAC GCA GCA TTC TTT ATT CAA TCA TTA ATA GAT TAT AGT AGC AAT Leu Asn Ala Ala Phe Phe Ile Gln Ser Leu Ile Asp Tyr Ser Ser Asn	2928
	965 970 975	
15	AAA GAT GTA CTG AAT GAT TTA AGT ACC TCA GTT AAG GTT CAA CTT TAT Lys Asp Val Leu Asn Asp Leu Ser Thr Ser Val Lys Val Gln Leu Tyr	2976
	980 985 990	
20	GCT CAA CTA TTT AGT ACA GGT TTA AAT ACT ATA TAT GAC TCT ATC CAA Ala Gln Leu Phe Ser Thr Gly Leu Asn Thr Ile Tyr Asp Ser Ile Gln	3024
	995 1000 1005	
	TTA GTA AAT TTA ATA TCA AAT GCA GTA AAT GAT ACT ATA AAT GTA CTA Leu Val Asn Leu Ile Ser Asn Ala Val Asn Asp Thr Ile Asn Val Leu	3072
	1010 1015 1020	
25	CCT ACA ATA ACA GAG GGG ATA CCT ATT GTA TCT ACT ATA TTA GAC GGA Pro Thr Ile Thr Glu Gly Ile Pro Ile Val Ser Thr Ile Leu Asp Gly	3120
	1025 1030 1035 1040	
30	ATA AAC TTA GGT GCA GCA ATT AAG GAA TTA CTA GAC GAA CAT GAC CCA Ile Asn Leu Gly Ala Ala Ile Lys Glu Leu Leu Asp Glu His Asp Pro	3168
	1045 1050 1055	
35	TTA CTA AAA AAA GAA TTA GAA GCT AAG GTG GGT GTT TTA GCA ATA AAT Leu Leu Lys Lys Glu Leu Glu Ala Lys Val Gly Val Leu Ala Ile Asn	3216
	1060 1065 1070	
40	ATG TCA TTA TCT ATA GCT GCA ACT GTA GCT TCA ATT GTT GGA ATA GGT Met Ser Leu Ser Ile Ala Ala Thr Val Ala Ser Ile Val Gly Ile Gly	3264
	1075 1080 1085	
	GCT GAA GTT ACT ATT TTC TTA TTA CCT ATA GCT GGT ATA TCT GCA GGA Ala Glu Val Thr Ile Phe Leu Leu Pro Ile Ala Gly Ile Ser Ala Gly	3312
	1090 1095 1100	
45	ATA CCT TCA TTA GTT AAT AAT GAA TTA ATA TTG CAT GAT AAG GCA ACT Ile Pro Ser Leu Val Asn Asn Glu Leu Ile Leu His Asp Lys Ala Thr	3360
	1105 1110 1115 1120	
50	TCA GTG GTA AAC TAT TTT AAT CAT TTG TCT GAA TCT AAA AAA TAT GGC Ser Val Val Asn Tyr Phe Asn His Leu Ser Glu Ser Lys Lys Tyr Gly	3408
	1125 1130 1135	
55	CCT CTT AAA ACA GAA GAT GAT AAA ATT TTA GTT CCT ATT GAT GAT TTA Pro Leu Lys Thr Glu Asp Asp Lys Ile Leu Val Pro Ile Asp Asp Leu	3456
	1140 1145 1150	
60	GTA ATA TCA GAA ATA GAT TTT AAT AAT AAT TCG ATA AAA CTA GGA ACA Val Ile Ser Glu Ile Asp Phe Asn Asn Asn Ser Ile Lys Leu Gly Thr	3504
	1155 1160 1165	
	TGT AAT ATA TTA GCA ATG GAG GGG GGA TCA GGA CAC ACA GTG ACT GGT Cys Asn Ile Leu Ala Met Glu Gly Gly Ser Gly His Thr Val Thr Gly	3552
	1170 1175 1180	
65	AAT ATA GAT CAC TTT TTC TCA TCT CCA TCT ATA AGT TCT CAT ATT CCT Asn Ile Asp His Phe Ser Ser Pro Ser Ile Ser Ser His Ile Pro	3600
	1185 1190 1195 1200	

	TCA TTA TCA ATT TAT TCT GCA ATA GGT ATA GAA ACA GAA AAT CTA GAT Ser Leu Ser Ile Tyr Ser Ala Ile Gly Ile Glu Thr Glu Asn Leu Asp 1205 1210 1215	3648
5	TTT TCA AAA AAA ATA ATG ATG TTA CCT AAT GCT CCT TCA AGA GTG TTT Phe Ser Lys Lys Ile Met Met Leu Pro Asn Ala Pro Ser Arg Val Phe 1220 1225 1230	3696
10	TGG TGG GAA ACT GGA GCA GTT CCA GGT TTA AGA TCA TTG GAA AAT GAC Trp Trp Glu Thr Gly Ala Val Pro Gly Leu Arg Ser Leu Glu Asn Asp 1235 1240 1245	3744
15	GGA ACT AGA TTA CTT GAT TCA ATA AGA GAT TTA TAC CCA GGT AAA TTT Gly Thr Arg Leu Leu Asp Ser Ile Arg Asp Leu Tyr Pro Gly Lys Phe 1250 1255 1260	3792
20	TAC TGG AGA TTC TAT GCT TTT TTC GAT TAT GCA ATA ACT ACA TTA AAA Tyr Trp Arg Phe Tyr Ala Phe Phe Asp Tyr Ala Ile Thr Thr Leu Lys 1265 1270 1275 1280	3840
	CCA GTT TAT GAA GAC ACT AAT ATT AAA ATT AAA CTA GAT AAA GAT ACT Pro Val Tyr Glu Asp Thr Asn Ile Lys Ile Lys Leu Asp Lys Asp Thr 1285 1290 1295	3888
25	AGA AAC TTC ATA ATG CCA ACT ATA ACT ACT AAC GAA ATT AGA AAC AAA Arg Asn Phe Ile Met Pro Thr Ile Thr Thr Asn Glu Ile Arg Asn Lys 1300 1305 1310	3936
30	TTA TCT TAT TCA TTT GAT GGA GCA GGA GGA ACT TAC TCT TTA TTA TTA Leu Ser Tyr Ser Phe Asp Gly Ala Gly Gly Thr Tyr Ser Leu Leu Leu 1315 1320 1325	3984
35	TCT TCA TAT CCA ATA TCA ACG AAT ATA AAT TTA TCT AAA GAT GAT TTA Ser Ser Tyr Pro Ile Ser Thr Asn Ile Asn Leu Ser Lys Asp Asp Leu 1330 1335 1340	4032
40	TGG ATA TTT AAT ATT GAT AAT GAA GTA AGA GAA ATA TCT ATA GAA AAT Trp Ile Phe Asn Ile Asp Asn Glu Val Arg Glu Ile Ser Ile Glu Asn 1345 1350 1355 1360	4080
	GGT ACT ATT AAA AAA GGA AAG TTA ATA AAA GAT GTT TTA AGT AAA ATT Gly Thr Ile Lys Lys Gly Lys Leu Ile Lys Asp Val Leu Ser Lys Ile 1365 1370 1375	4128
45	GAT ATA AAT AAA AAT AAA CTT ATT ATA GGC AAT CAA ACA ATA GAT TTT Asp Ile Asn Lys Asn Lys Leu Ile Ile Gly Asn Gln Thr Ile Asp Phe 1380 1385 1390	4176
50	TCA GGC GAT ATA GAT AAT AAA GAT AGA TAT ATA TTC TTG ACT TGT GAG Ser Gly Asp Ile Asp Asn Lys Asp Arg Tyr Ile Phe Leu Thr Cys Glu 1395 1400 1405	4224
55	TTA GAT GAT AAA ATT AGT TTA ATA ATA GAA ATA AAT CTT GTT GCA AAA Leu Asp Asp Lys Ile Ser Leu Ile Ile Glu Ile Asn Leu Val Ala Lys 1410 1415 1420	4272
60	TCT TAT AGT TTG TTA TTG TCT GGG GAT AAA AAT TAT TTG ATA TCC AAT Ser Tyr Ser Leu Leu Leu Ser Gly Asp Lys Asn Tyr Leu Ile Ser Asn 1425 1430 1435 1440	4320
	TTA TCT AAT ACT ATT GAG AAA ATC AAT ACT TTA GGC CTA GAT AGT AAA Leu Ser Asn Thr Ile Glu Lys Ile Asn Thr Leu Gly Leu Asp Ser Lys 1445 1450 1455	4368

	AAT ATA GCG TAC AAT TAC ACT GAT GAA TCT AAT AAT AAA TAT TTT GGA Asn Ile Ala Tyr Asn Tyr Thr Asp Glu Ser Asn Asn Lys Tyr Phe Gly 1460 1465 1470	4416
5	GCT ATA TCT AAA ACA AGT CAA AAA AGC ATA ATA CAT TAT AAA AAA GAC Ala Ile Ser Lys Thr Ser Gln Lys Ser Ile Ile His Tyr Lys Lys Asp 1475 1480 1485	4464
10	AGT AAA AAT ATA TTA GAA TTT TAT AAT GAC AGT ACA TTA GAA TTT AAC Ser Lys Asn Ile Leu Glu Phe Tyr Asn Asp Ser Thr Leu Glu Phe Asn 1490 1495 1500	4512
15	AGT AAA GAT TTT ATT GCT GAA GAT ATA AAT GTA TTT ATG AAA GAT GAT Ser Lys Asp Phe Ile Ala Glu Asp Ile Asn Val Phe Met Lys Asp Asp 1505 1510 1515 1520	4560
20	ATT AAT ACT ATA ACA GGA AAA TAC TAT GTT GAT AAT AAT ACT GAT AAA Ile Asn Thr Ile Thr Gly Lys Tyr Tyr Val Asp Asn Asn Thr Asp Lys 1525 1530 1535	4608
	AGT ATA GAT TTC TCT ATT TCT TTA GTT AGT AAA AAT CAA GTA AAA GTA Ser Ile Asp Phe Ser Ile Ser Leu Val Ser Lys Asn Gln Val Lys Val 1540 1545 1550	4656
25	AAT GGA TTA TAT TTA AAT GAA TCC GTA TAC TCA TCT TAC CTT GAT TTT Asn Gly Leu Tyr Leu Asn Glu Ser Val Tyr Ser Ser Tyr Leu Asp Phe 1555 1560 1565	4704
30	GTG AAA AAT TCA GAT GGA CAC CAT AAT ACT TCT AAT TTT ATG AAT TTA Val Lys Asn Ser Asp Gly His His Asn Thr Ser Asn Phe Met Asn Leu 1570 1575 1580	4752
35	TTT TTG GAC AAT ATA AGT TTC TGG AAA TTG TTT GGG TTT GAA AAT ATA Phe Leu Asp Asn Ile Ser Phe Trp Lys Leu Phe Gly Phe Glu Asn Ile 1585 1590 1595 1600	4800
40	AAT TTT GTA ATC GAT AAA TAC TTT ACC CTT GTT GGT AAA ACT AAT CTT Asn Phe Val Ile Asp Lys Tyr Phe Thr Leu Val Gly Lys Thr Asn Leu 1605 1610 1615	4848
	GGA TAT GTA GAA TTT ATT TGT GAC AAT AAT AAA AAT ATA GAT ATA TAT Gly Tyr Val Glu Phe Ile Cys Asp Asn Asn Lys Asn Ile Asp Ile Tyr 1620 1625 1630	4896
45	TTT GGT GAA TGG AAA ACA TCG TCA TCT AAA AGC ACT ATA TTT AGC GGA Phe Gly Glu Trp Lys Thr Ser Ser Ser Lys Ser Thr Ile Phe Ser Gly 1635 1640 1645	4944
50	AAT GGT AGA AAT GTT GTA GTA GAG CCT ATA TAT AAT CCT GAT ACG GGT Asn Gly Arg Asn Val Val Val Glu Pro Ile Tyr Asn Pro Asp Thr Gly 1650 1655 1660	4992
55	GAA GAT ATA TCT ACT TCA CTA GAT TTT TCC TAT GAA CCT CTC TAT GGA Glu Asp Ile Ser Thr Ser Leu Asp Phe Ser Tyr Glu Pro Leu Tyr Gly 1665 1670 1675 1680	5040
60	ATA GAT AGA TAT ATA AAT AAA GTA TTG ATA GCA CCT GAT TTA TAT ACA Ile Asp Arg Tyr Ile Asn Lys Val Leu Ile Ala Pro Asp Leu Tyr Thr 1685 1690 1695	5088
	AGT TTA ATA AAT ATT AAT ACC AAT TAT TAT TCA AAT GAG TAC TAC CCT Ser Leu Ile Asn Ile Asn Thr Asn Tyr Tyr Ser Asn Glu Tyr Tyr Pro 1700 1705 1710	5136
65	GAG ATT ATA GTT CTT AAC CCA AAT ACA TTC CAC AAA AAA GTA AAT ATA Glu Ile Ile Val Leu Asn Pro Asn Thr Phe His Lys Lys Val Asn Ile 1715 1720 1725	5184



	AAT TTA GAT AGT TCT TCT TTT GAG TAT AAA TGG TCT ACA GAA GGA AGT Asn Leu Asp Ser Ser Ser Phe Glu Tyr Lys Trp Ser Thr Glu Gly Ser 1730 1735 1740	5232
5	GAC TTT ATT TTA GTT AGA TAC TTA GAA GAA AGT AAT AAA AAA ATA TTA Asp Phe Ile Leu Val Arg Tyr Leu Glu Glu Ser Asn Lys Lys Ile Leu 1745 1750 1755 1760	5280
10	CAA AAA ATA AGA ATC AAA GGT ATC TTA TCT AAT ACT CAA TCA TTT AAT Gln Lys Ile Arg Ile Lys Gly Ile Leu Ser Asn Thr Gln Ser Phe Asn 1765 1770 1775	5328
15	AAA ATG AGT ATA GAT TTT AAA GAT ATT AAA AAA CTA TCA TTA GGA TAT Lys Met Ser Ile Asp Phe Lys Asp Ile Lys Lys Leu Ser Leu Gly Tyr 1780 1785 1790	5376
20	ATA ATG AGT AAT TTT AAA TCA TTT AAT TCT GAA AAT GAA TTA GAT AGA Ile Met Ser Asn Phe Lys Ser Phe Asn Ser Glu Asn Glu Leu Asp Arg 1795 1800 1805	5424
	GAT CAT TTA GGA TTT AAA ATA ATA GAT AAT AAA ACT TAT TAC TAT GAT Asp His Leu Gly Phe Lys Ile Ile Asp Asn Lys Thr Tyr Tyr Tyr Asp 1810 1815 1820	5472
25	GAA GAT AGT AAA TTA GTT AAA GGA TTA ATC AAT ATA AAT AAT TCA TTA Glu Asp Ser Lys Leu Val Lys Gly Leu Ile Asn Ile Asn Asn Ser Leu 1825 1830 1835 1840	5520
30	TTC TAT TTT GAT CCT ATA GAA TTT AAC TTA GTA ACT GGA TGG CAA ACT Phe Tyr Phe Asp Pro Ile Glu Phe Asn Leu Val Thr Gly Trp Gln Thr 1845 1850 1855	5568
35	ATC AAT GGT AAA AAA TAT TAT TTT GAT ATA AAT ACT GGA GCA GCT TTA Ile Asn Gly Lys Lys Tyr Tyr Phe Asp Ile Asn Thr Gly Ala Ala Leu 1860 1865 1870	5616
	ACT AGT TAT AAA ATT ATT AAT GGT AAA CAC TTT TAT TTT AAT AAT GAT Thr Ser Tyr Lys Ile Ile Asn Gly Lys His Phe Tyr Phe Asn Asn Asp 1875 1880 1885	5664
40	GGT GTG ATG CAG TTG GGA GTA TTT AAA GGA CCT GAT GGA TTT GAA TAT Gly Val Met Gln Leu Gly Val Phe Lys Gly Pro Asp Gly Phe Glu Tyr 1890 1895 1900	5712
45	TTT GCA CCT GCC AAT ACT CAA AAT AAT AAC ATA GAA GGT CAG GCT ATA Phe Ala Pro Ala Asn Thr Gln Asn Asn Asn Ile Glu Gly Gln Ala Ile 1905 1910 1915 1920	5760
50	GTT TAT CAA AGT AAA TTC TTA ACT TTG AAT GGC AAA AAA TAT TAT TTT Val Tyr Gln Ser Lys Phe Leu Thr Leu Asn Gly Lys Lys Tyr Tyr Phe 1925 1930 1935	5808
55	GAT AAT AAC TCA AAA GCA GTC ACT GGA TGG AGA ATT ATT AAC AAT GAG Asp Asn Asn Ser Lys Ala Val Thr Gly Trp Arg Ile Ile Asn Asn Glu 1940 1945 1950	5856
	AAA TAT TAC TTT AAT CCT AAT AAT GCT ATT GCT GCA GTC GGA TTG CAA Lys Tyr Tyr Phe Asn Pro Asn Asn Ala Ile Ala Ala Val Gly Leu Gln 1955 1960 1965	5904
60	GTA ATT GAC AAT AAT AAG TAT TAT TTC AAT CCT GAC ACT GCT ATC ATC Val Ile Asp Asn Asn Lys Tyr Tyr Phe Asn Pro Asp Thr Ala Ile Ile 1970 1975 1980	5952
65	TCA AAA GGT TGG CAG ACT GTT AAT GGT AGT AGA TAC TAC TTT GAT ACT Ser Lys Gly Trp Gln Thr Val Asn Gly Ser Arg Tyr Tyr Phe Asp Thr 1985 1990 1995 2000	6000

	GAT ACC GCT ATT GCC TTT AAT GGT TAT AAA ACT ATT GAT GGT AAA CAC Asp Thr Ala Ile Ala Phe Asn Gly Tyr Lys Thr Ile Asp Gly Lys His 2005 2010 2015	6048
5	TTT TAT TTT GAT AGT GAT TGT GTA GTG AAA ATA GGT GTG TTT AGT ACC Phe Tyr Phe Asp Ser Asp Cys Val Val Lys Ile Gly Val Phe Ser Thr 2020 2025 2030	6096
10	TCT AAT GGA TTT GAA TAT TTT GCA CCT GCT AAT ACT TAT AAT AAT AAC Ser Asn Gly Phe Glu Tyr Phe Ala Pro Ala Asn Thr Tyr Asn Asn Asn 2035 2040 2045	6144
15	ATA GAA GGT CAG GCT ATA GTT TAT CAA AGT AAA TTC TTA ACT TTG AAT Ile Glu Gly Gln Ala Ile Val Tyr Gln Ser Lys Phe Leu Thr Leu Asn 2050 2055 2060	6192
20	GGT AAA AAA TAT TAC TTT GAT AAT AAC TCA AAA GCA GTT ACC GGA TTG Gly Lys Lys Tyr Tyr Phe Asp Asn Asn Ser Lys Ala Val Thr Gly Leu 2065 2070 2075 2080	6240
	CAA ACT ATT GAT AGT AAA AAA TAT TAC TTT AAT ACT AAC ACT GCT GAA Gln Thr Ile Asp Ser Lys Lys Tyr Tyr Phe Asn Thr Asn Thr Ala Glu 2085 2090 2095	6288
25	GCA GCT ACT GGA TGG CAA ACT ATT GAT GGT AAA AAA TAT TAC TTT AAT Ala Ala Thr Gly Trp Gln Thr Ile Asp Gly Lys Lys Tyr Tyr Phe Asn 2100 2105 2110	6336
30	ACT AAC ACT GCT GAA GCA GCT ACT GGA TGG CAA ACT ATT GAT GGT AAA Thr Asn Thr Ala Glu Ala Ala Thr Gly Trp Gln Thr Ile Asp Gly Lys 2115 2120 2125	6384
35	AAA TAT TAC TTT AAT ACT AAC ACT GCT ATA GCT TCA ACT GGT TAT ACA Lys Tyr Tyr Phe Asn Thr Asn Thr Ala Ile Ala Ser Thr Gly Tyr Thr 2130 2135 2140	6432
40	ATT ATT AAT GGT AAA CAT TTT TAT TTT AAT ACT GAT GGT ATT ATG CAG Ile Ile Asn Gly Lys His Phe Tyr Phe Asn Thr Asp Gly Ile Met Gln 2145 2150 2155 2160	6480
	ATA GGA GTG TTT AAA GGA CCT AAT GGA TTT GAA TAT TTT GCA CCT GCT Ile Gly Val Phe Lys Gly Pro Asn Gly Phe Glu Tyr Phe Ala Pro Ala 2165 2170 2175	6528
45	AAT ACG GAT GCT AAC AAC ATA GAA GGT CAA GCT ATA CTT TAC CAA AAT Asn Thr Asp Ala Asn Asn Ile Glu Gly Gln Ala Ile Leu Tyr Gln Asn 2180 2185 2190	6576
50	GAA TTC TTA ACT TTG AAT GGT AAA AAA TAT TAC TTT GGT AGT GAC TCA Glu Phe Leu Thr Leu Asn Gly Lys Lys Tyr Tyr Phe Gly Ser Asp Ser 2195 2200 2205	6624
55	AAA GCA GTT ACT GGA TGG AGA ATT ATT AAC AAT AAG AAA TAT TAC TTT Lys Ala Val Thr Gly Trp Arg Ile Ile Asn Asn Lys Lys Tyr Tyr Phe 2210 2215 2220	6672
60	AAT CCT AAT AAT GCT ATT GCT GCA ATT CAT CTA TGC ACT ATA AAT AAT Asn Pro Asn Asn Ala Ile Ala Ala Ile His Leu Cys Thr Ile Asn Asn 2225 2230 2235 2240	6720
	GAC AAG TAT TAC TTT AGT TAT GAT GGA ATT CTT CAA AAT GGA TAT ATT Asp Lys Tyr Tyr Phe Ser Tyr Asp Gly Ile Leu Gln Asn Gly Tyr Ile 2245 2250 2255	6768
65	ACT ATT GAA AGA AAT AAT TTC TAT TTT GAT GCT AAT AAT GAA TCT AAA Thr Ile Glu Arg Asn Asn Phe Tyr Phe Asp Ala Asn Asn Glu Ser Lys 2260 2265 2270	6816

	ATG GTA ACA GGA GTA TTT AAA GGA CCT AAT GGA TTT GAG TAT TTT GCA Met Val Thr Gly Val Phe Lys Gly Pro Asn Gly Phe Glu Tyr Phe Ala 2275 2280 2285	6864
5	CCT GCT AAT ACT CAC AAT AAT AAC ATA GAA GGT CAG GCT ATA GTT TAC Pro Ala Asn Thr His Asn Asn Asn Ile Glu Gly Gln Ala Ile Val Tyr 2290 2295 2300	6912
10	CAG AAC AAA TTC TTA ACT TTG AAT GGC AAA AAA TAT TAT TTT GAT AAT Gln Asn Lys Phe Leu Thr Leu Asn Gly Lys Lys Tyr Tyr Phe Asp Asn 2305 2310 2315 2320	6960
15	GAC TCA AAA GCA GTT ACT GGA TGG CAA ACC ATT GAT GGT AAA AAA TAT Asp Ser Lys Ala Val Thr Gly Trp Gln Thr Ile Asp Gly Lys Lys Tyr 2325 2330 2335	7008
20	TAC TTT AAT CTT AAC ACT GCT GAA GCA GCT ACT GGA TGG CAA ACT ATT Tyr Phe Asn Leu Asn Thr Ala Glu Ala Ala Thr Gly Trp Gln Thr Ile 2340 2345 2350	7056
	GAT GGT AAA AAA TAT TAC TTT AAT CTT AAC ACT GCT GAA GCA GCT ACT Asp Gly Lys Lys Tyr Tyr Phe Asn Leu Asn Thr Ala Glu Ala Ala Thr 2355 2360 2365	7104
25	GGA TGG CAA ACT ATT GAT GGT AAA AAA TAT TAC TTT AAT ACT AAC ACT Gly Trp Gln Thr Ile Asp Gly Lys Lys Tyr Tyr Phe Asn Thr Asn Thr 2370 2375 2380	7152
30	TTC ATA GCC TCA ACT GGT TAT ACA AGT ATT AAT GGT AAA CAT TTT TAT Phe Ile Ala Ser Thr Gly Tyr Thr Ser Ile Asn Gly Lys His Phe Tyr 2385 2390 2395 2400	7200
35	TTT AAT ACT GAT GGT ATT ATG CAG ATA GGA GTG TTT AAA GGA CCT AAT Phe Asn Thr Asp Gly Ile Met Gln Ile Gly Val Phe Lys Gly Pro Asn 2405 2410 2415	7248
40	GGA TTT GAA TAC TTT GCA CCT GCT AAT ACG GAT GCT AAC AAC ATA GAA Gly Phe Glu Tyr Phe Ala Pro Ala Asn Thr Asp Ala Asn Asn Ile Glu 2420 2425 2430	7296
	GGT CAA GCT ATA CTT TAC CAA AAT AAA TTC TTA ACT TTG AAT GGT AAA Gly Gln Ala Ile Leu Tyr Gln Asn Lys Phe Leu Thr Leu Asn Gly Lys 2435 2440 2445	7344
45	AAA TAT TAC TTT GGT AGT GAC TCA AAA GCA GTT ACC GGA CTG CGA ACT Lys Tyr Tyr Phe Gly Ser Asp Ser Lys Ala Val Thr Gly Leu Arg Thr 2450 2455 2460	7392
50	ATT GAT GGT AAA AAA TAT TAC TTT AAT ACT AAC ACT GCT GTT GCA GTT Ile Asp Gly Lys Lys Tyr Tyr Phe Asn Thr Asn Thr Ala Val Ala Val 2465 2470 2475 2480	7440
55	ACT GGA TGG CAA ACT ATT AAT GGT AAA AAA TAC TAC TTT AAT ACT AAC Thr Gly Trp Gln Thr Ile Asn Gly Lys Lys Tyr Tyr Phe Asn Thr Asn 2485 2490 2495	7488
60	ACT TCT ATA GCT TCA ACT GGT TAT ACA ATT ATT AGT GGT AAA CAT TTT Thr Ser Ile Ala Ser Thr Gly Tyr Thr Ile Ile Ser Gly Lys His Phe 2500 2505 2510	7536
	TAT TTT AAT ACT GAT GGT ATT ATG CAG ATA GGA GTG TTT AAA GGA CCT Tyr Phe Asn Thr Asp Gly Ile Met Gln Ile Gly Val Phe Lys Gly Pro 2515 2520 2525	7584
65	GAT GGA TTT GAA TAC TTT GCA CCT GCT AAT ACA GAT GCT AAC AAT ATA Asp Gly Phe Glu Tyr Phe Ala Pro Ala Asn Thr Asp Ala Asn Asn Ile 2530 2535 2540	7632

	GAA GGT CAA GCT ATA CGT TAT CAA AAT AGA TTC CTA TAT TTA CAT GAC Glu Gly Gln Ala Ile Arg Tyr Gln Asn Arg Phe Leu Tyr Leu His Asp 2545 2550 2555 2560	7680
5	AAT ATA TAT TAT TTT GGT AAT AAT TCA AAA GCG GCT ACT GGT TGG GTA Asn Ile Tyr Tyr Phe Gly Asn Asn Ser Lys Ala Ala Thr Gly Trp Val 2565 2570 2575	7728
10	ACT ATT GAT GGT AAT AGA TAT TAC TTC GAG CCT AAT ACA GCT ATG GGT Thr Ile Asp Gly Asn Arg Tyr Tyr Phe Glu Pro Asn Thr Ala Met Gly 2580 2585 2590	7776
15	GCG AAT GGT TAT AAA ACT ATT GAT AAT AAA AAT TTT TAC TTT AGA AAT Ala Asn Gly Tyr Lys Thr Ile Asp Asn Lys Asn Phe Tyr Phe Arg Asn 2595 2600 2605	7824
20	GGT TTA CCT CAG ATA GGA GTG TTT AAA GGG TCT AAT GGA TTT GAA TAC Gly Leu Pro Gln Ile Gly Val Phe Lys Gly Ser Asn Gly Phe Glu Tyr 2610 2615 2620	7872
	TTT GCA CCT GCT AAT ACG GAT GCT AAC AAT ATA GAA GGT CAA GCT ATA Phe Ala Pro Ala Asn Thr Asp Ala Asn Asn Ile Glu Gly Gln Ala Ile 2625 2630 2635 2640	7920
25	CGT TAT CAA AAT AGA TTC CTA CAT TTA CTT GGA AAA ATA TAT TAC TTT Arg Tyr Gln Asn Arg Phe Leu His Leu Leu Gly Lys Ile Tyr Tyr Phe 2645 2650 2655	7968
30	GGT AAT AAT TCA AAA GCA GTT ACT GGA TGG CAA ACT ATT AAT GGT AAA Gly Asn Asn Ser Lys Ala Val Thr Gly Trp Gln Thr Ile Asn Gly Lys 2660 2665 2670	8016
35	GTA TAT TAC TTT ATG CCT GAT ACT GCT ATG GCT GCA GCT GGT GGA CTT Val Tyr Tyr Phe Met Pro Asp Thr Ala Met Ala Ala Ala Gly Gly Leu 2675 2680 2685	8064
40	TTC GAG ATT GAT GGT GTT ATA TAT TTC TTT GGT GTT GAT GGA GTA AAA Phe Glu Ile Asp Gly Val Ile Tyr Phe Phe Gly Val Asp Gly Val Lys 2690 2695 2700	8112
	GCC CCT GGG ATA TAT GGC TAA Ala Pro Gly Ile Tyr Gly 2705 2710	8133
45	(2) INFORMATION FOR SEQ ID NO:6:	
	(i) SEQUENCE CHARACTERISTICS:	
	(A) LENGTH: 2710 amino acids	
50	(B) TYPE: amino acid	
	(D) TOPOLOGY: linear	
	(ii) MOLECULE TYPE: protein	
55	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:	
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60	Arg Pro Arg Glu Asn Glu Tyr Lys Thr Ile Leu Thr Asn Leu Asp Glu 20 25 30	
	Tyr Asn Lys Leu Thr Thr Asn Asn Asn Glu Asn Lys Tyr Leu Gln Leu 35 40 45	
65	Lys Lys Leu Asn Glu Ser Ile Asp Val Phe Met Asn Lys Tyr Lys Thr 50 55 60	
70	Ser Ser Arg Asn Arg Ala Leu Ser Asn Leu Lys Lys Asp Ile Leu Lys 65 70 75 80	

- 231 -

Ile Ala Pro Tyr Leu Gln Val Gly Phe Met Pro Glu Ala Arg Ser Thr  
 450 455 460  
 5 Ile Ser Leu Ser Gly Pro Gly Ala Tyr Ala Ser Ala Tyr Tyr Asp Phe  
 465 470 475 480  
 Ile Asn Leu Gln Glu Asn Thr Ile Glu Lys Thr Leu Lys Ala Ser Asp  
 485 490 495  
 10 Leu Ile Glu Phe Lys Phe Pro Glu Asn Asn Leu Ser Gln Leu Thr Glu  
 500 505 510  
 Gln Glu Ile Asn Ser Leu Trp Ser Phe Asp Gln Ala Ser Ala Lys Tyr  
 515 520 525  
 15 Gln Phe Glu Lys Tyr Val Arg Asp Tyr Thr Gly Gly Ser Leu Ser Glu  
 530 535 540  
 Asp Asn Gly Val Asp Phe Asn Lys Asn Thr Ala Leu Asp Lys Asn Tyr  
 545 550 555 560  
 Leu Leu Asn Asn Lys Ile Pro Ser Asn Asn Val Glu Glu Ala Gly Ser  
 565 570 575  
 25 Lys Asn Tyr Val His Tyr Ile Ile Gln Leu Gln Gly Asp Asp Ile Ser  
 580 585 590  
 Tyr Glu Ala Thr Cys Asn Leu Phe Ser Lys Asn Pro Lys Asn Ser Ile  
 595 600 605  
 30 Ile Ile Gln Arg Asn Met Asn Glu Ser Ala Lys Ser Tyr Phe Leu Ser  
 610 615 620  
 Asp Asp Gly Glu Ser Ile Leu Glu Leu Asn Lys Tyr Arg Ile Pro Glu  
 625 630 635 640  
 Arg Leu Lys Asn Lys Glu Lys Val Lys Val Thr Phe Ile Gly His Gly  
 645 650 655  
 40 Lys Asp Glu Phe Asn Thr Ser Glu Phe Ala Arg Leu Ser Val Asp Ser  
 660 665 670  
 Leu Ser Asn Glu Ile Ser Ser Phe Leu Asp Thr Ile Lys Leu Asp Ile  
 675 680 685  
 45 Ser Pro Lys Asn Val Glu Val Asn Leu Leu Gly Cys Asn Met Phe Ser  
 690 695 700  
 Tyr Asp Phe Asn Val Glu Glu Thr Tyr Pro Gly Lys Leu Leu Leu Ser  
 705 710 715 720  
 Ile Met Asp Lys Ile Thr Ser Thr Leu Pro Asp Val Asn Lys Asn Ser  
 725 730 735  
 55 Ile Thr Ile Gly Ala Asn Gln Tyr Glu Val Arg Ile Asn Ser Glu Gly  
 740 745 750  
 Arg Lys Glu Leu Leu Ala His Ser Gly Lys Trp Ile Asn Lys Glu Glu  
 755 760 765  
 60 Ala Ile Met Ser Asp Leu Ser Ser Lys Glu Tyr Ile Phe Phe Asp Ser  
 770 775 780  
 Ile Asp Asn Lys Leu Lys Ala Lys Ser Lys Asn Ile Pro Gly Leu Ala  
 785 790 795 800  
 65 Ser Ile Ser Glu Asp Ile Lys Thr Leu Leu Leu Asp Ala Ser Val Ser  
 805 810 815

Pro Asp Thr Lys Phe Ile Leu Asn Asn Leu Lys Leu Asn Ile Glu Ser  
                     820                    825                    830  
 5 Ser Ile Gly Asp Tyr Ile Tyr Tyr Glu Lys Leu Glu Pro Val Lys Asn  
                     835                    840                    845  
 Ile Ile His Asn Ser Ile Asp Asp Leu Ile Asp Glu Phe Asn Leu Leu  
                     850                    855                    860  
 10 Glu Asn Val Ser Asp Glu Leu Tyr Glu Leu Lys Lys Leu Asn Asn Leu  
                     865                    870                    875                    880  
 Asp Glu Lys Tyr Leu Ile Ser Phe Glu Asp Ile Ser Lys Asn Asn Ser  
                     885                    890                    895  
 15 Thr Tyr Ser Val Arg Phe Ile Asn Lys Ser Asn Gly Glu Ser Val Tyr  
                     900                    905                    910  
 20 Val Glu Thr Glu Lys Glu Ile Phe Ser Lys Tyr Ser Glu His Ile Thr  
                     915                    920                    925  
 Lys Glu Ile Ser Thr Ile Lys Asn Ser Ile Ile Thr Asp Val Asn Gly  
                     930                    935                    940  
 25 Asn Leu Leu Asp Asn Ile Gln Leu Asp His Thr Ser Gln Val Asn Thr  
                     945                    950                    955                    960  
 Leu Asn Ala Ala Phe Phe Ile Gln Ser Leu Ile Asp Tyr Ser Ser Asn  
                     965                    970                    975  
 30 Lys Asp Val Leu Asn Asp Leu Ser Thr Ser Val Lys Val Gln Leu Tyr  
                     980                    985                    990  
 35 Ala Gln Leu Phe Ser Thr Gly Leu Asn Thr Ile Tyr Asp Ser Ile Gln  
                     995                    1000                    1005  
 Leu Val Asn Leu Ile Ser Asn Ala Val Asn Asp Thr Ile Asn Val Leu  
                     1010                    1015                    1020  
 40 Pro Thr Ile Thr Glu Gly Ile Pro Ile Val Ser Thr Ile Leu Asp Gly  
                     1025                    1030                    1035                    1040  
 Ile Asn Leu Gly Ala Ala Ile Lys Glu Leu Leu Asp Glu His Asp Pro  
                     1045                    1050                    1055  
 45 Leu Leu Lys Lys Glu Leu Glu Ala Lys Val Gly Val Leu Ala Ile Asn  
                     1060                    1065                    1070  
 50 Met Ser Leu Ser Ile Ala Ala Thr Val Ala Ser Ile Val Gly Ile Gly  
                     1075                    1080                    1085  
 Ala Glu Val Thr Ile Phe Leu Leu Pro Ile Ala Gly Ile Ser Ala Gly  
                     1090                    1095                    1100  
 55 Ile Pro Ser Leu Val Asn Asn Glu Leu Ile Leu His Asp Lys Ala Thr  
                     1105                    1110                    1115                    1120  
 Ser Val Val Asn Tyr Phe Asn His Leu Ser Glu Ser Lys Lys Tyr Gly  
                     1125                    1130                    1135  
 60 Pro Leu Lys Thr Glu Asp Asp Lys Ile Leu Val Pro Ile Asp Asp Leu  
                     1140                    1145                    1150  
 65 Val Ile Ser Glu Ile Asp Phe Asn Asn Asn Ser Ile Lys Leu Gly Thr  
                     1155                    1160                    1165  
 Cys Asn Ile Leu Ala Met Glu Gly Gly Ser Gly His Thr Val Thr Gly  
                     1170                    1175                    1180

Asn Ile Asp His Phe Phe Ser Ser Pro Ser Ile Ser Ser His Ile Pro  
 1185 1190 1195 1200  
 5 Ser Leu Ser Ile Tyr Ser Ala Ile Gly Ile Glu Thr Glu Asn Leu Asp  
 1205 1210 1215  
 Phe Ser Lys Lys Ile Met Met Leu Pro Asn Ala Pro Ser Arg Val Phe  
 1220 1225 1230  
 10 Trp Trp Glu Thr Gly Ala Val Pro Gly Leu Arg Ser Leu Glu Asn Asp  
 1235 1240 1245  
 Gly Thr Arg Leu Leu Asp Ser Ile Arg Asp Leu Tyr Pro Gly Lys Phe  
 1250 1255 1260  
 15 Tyr Trp Arg Phe Tyr Ala Phe Phe Asp Tyr Ala Ile Thr Thr Leu Lys  
 1265 1270 1275 1280  
 20 Pro Val Tyr Glu Asp Thr Asn Ile Lys Ile Lys Leu Asp Lys Asp Thr  
 1285 1290 1295  
 Arg Asn Phe Ile Met Pro Thr Ile Thr Thr Asn Glu Ile Arg Asn Lys  
 1300 1305 1310  
 25 Leu Ser Tyr Ser Phe Asp Gly Ala Gly Gly Thr Tyr Ser Leu Leu Leu  
 1315 1320 1325  
 Ser Ser Tyr Pro Ile Ser Thr Asn Ile Asn Leu Ser Lys Asp Asp Leu  
 1330 1335 1340  
 30 Trp Ile Phe Asn Ile Asp Asn Glu Val Arg Glu Ile Ser Ile Glu Asn  
 1345 1350 1355 1360  
 35 Gly Thr Ile Lys Lys Gly Lys Leu Ile Lys Asp Val Leu Ser Lys Ile  
 1365 1370 1375  
 Asp Ile Asn Lys Asn Lys Leu Ile Ile Gly Asn Gln Thr Ile Asp Phe  
 1380 1385 1390  
 40 Ser Gly Asp Ile Asp Asn Lys Asp Arg Tyr Ile Phe Leu Thr Cys Glu  
 1395 1400 1405  
 Leu Asp Asp Lys Ile Ser Leu Ile Ile Glu Ile Asn Leu Val Ala Lys  
 1410 1415 1420  
 45 Ser Tyr Ser Leu Leu Leu Ser Gly Asp Lys Asn Tyr Leu Ile Ser Asn  
 1425 1430 1435 1440  
 50 Leu Ser Asn Thr Ile Glu Lys Ile Asn Thr Leu Gly Leu Asp Ser Lys  
 1445 1450 1455  
 Asn Ile Ala Tyr Asn Tyr Thr Asp Glu Ser Asn Asn Lys Tyr Phe Gly  
 1460 1465 1470  
 55 Ala Ile Ser Lys Thr Ser Gln Lys Ser Ile Ile His Tyr Lys Lys Asp  
 1475 1480 1485  
 Ser Lys Asn Ile Leu Glu Phe Tyr Asn Asp Ser Thr Leu Glu Phe Asn  
 1490 1495 1500  
 60 Ser Lys Asp Phe Ile Ala Glu Asp Ile Asn Val Phe Met Lys Asp Asp  
 1505 1510 1515 1520  
 65 Ile Asn Thr Ile Thr Gly Lys Tyr Tyr Val Asp Asn Asn Thr Asp Lys  
 1525 1530 1535  
 Ser Ile Asp Phe Ser Ile Ser Leu Val Ser Lys Asn Gln Val Lys Val  
 1540 1545 1550



Asn Gly Leu Tyr Leu Asn Glu Ser Val Tyr Ser Ser Tyr Leu Asp Phe  
 1555 1560 1565  
 5 Val Lys Asn Ser Asp Gly His His Asn Thr Ser Asn Phe Met Asn Leu  
 1570 1575 1580  
 Phe Leu Asp Asn Ile Ser Phe Trp Lys Leu Phe Gly Phe Glu Asn Ile  
 1585 1590 1595 1600  
 10 Asn Phe Val Ile Asp Lys Tyr Phe Thr Leu Val Gly Lys Thr Asn Leu  
 1605 1610 1615  
 Gly Tyr Val Glu Phe Ile Cys Asp Asn Asn Lys Asn Ile Asp Ile Tyr  
 1620 1625 1630  
 15 Phe Gly Glu Trp Lys Thr Ser Ser Ser Lys Ser Thr Ile Phe Ser Gly  
 1635 1640 1645  
 20 Asn Gly Arg Asn Val Val Val Glu Pro Ile Tyr Asn Pro Asp Thr Gly  
 1650 1655 1660  
 Glu Asp Ile Ser Thr Ser Leu Asp Phe Ser Tyr Glu Pro Leu Tyr Gly  
 1665 1670 1675 1680  
 25 Ile Asp Arg Tyr Ile Asn Lys Val Leu Ile Ala Pro Asp Leu Tyr Thr  
 1685 1690 1695  
 Ser Leu Ile Asn Ile Asn Thr Asn Tyr Tyr Ser Asn Glu Tyr Tyr Pro  
 1700 1705 1710  
 30 Glu Ile Ile Val Leu Asn Pro Asn Thr Phe His Lys Lys Val Asn Ile  
 1715 1720 1725  
 35 Asn Leu Asp Ser Ser Ser Phe Glu Tyr Lys Trp Ser Thr Glu Gly Ser  
 1730 1735 1740  
 Asp Phe Ile Leu Val Arg Tyr Leu Glu Glu Ser Asn Lys Lys Ile Leu  
 1745 1750 1755 1760  
 40 Gln Lys Ile Arg Ile Lys Gly Ile Leu Ser Asn Thr Gln Ser Phe Asn  
 1765 1770 1775  
 Lys Met Ser Ile Asp Phe Lys Asp Ile Lys Lys Leu Ser Leu Gly Tyr  
 1780 1785 1790  
 45 Ile Met Ser Asn Phe Lys Ser Phe Asn Ser Glu Asn Glu Leu Asp Arg  
 1795 1800 1805  
 50 Asp His Leu Gly Phe Lys Ile Ile Asp Asn Lys Thr Tyr Tyr Tyr Asp  
 1810 1815 1820  
 Glu Asp Ser Lys Leu Val Lys Gly Leu Ile Asn Ile Asn Asn Ser Leu  
 1825 1830 1835 1840  
 55 Phe Tyr Phe Asp Pro Ile Glu Phe Asn Leu Val Thr Gly Trp Gln Thr  
 1845 1850 1855  
 Ile Asn Gly Lys Lys Tyr Tyr Phe Asp Ile Asn Thr Gly Ala Ala Leu  
 1860 1865 1870  
 60 Thr Ser Tyr Lys Ile Ile Asn Gly Lys His Phe Tyr Phe Asn Asn Asp  
 1875 1880 1885  
 65 Gly Val Met Gln Leu Gly Val Phe Lys Gly Pro Asp Gly Phe Glu Tyr  
 1890 1895 1900  
 Phe Ala Pro Ala Asn Thr Gln Asn Asn Asn Ile Glu Gly Gln Ala Ile  
 1905 1910 1915 1920

Val Tyr Gln Ser Lys Phe Leu Thr Leu Asn Gly Lys Lys Tyr Tyr Phe  
 1925 1930 1935  
 5 Asp Asn Asn Ser Lys Ala Val Thr Gly Trp Arg Ile Ile Asn Asn Glu  
 1940 1945 1950  
 Lys Tyr Tyr Phe Asn Pro Asn Asn Ala Ile Ala Ala Val Gly Leu Gln  
 1955 1960 1965  
 10 Val Ile Asp Asn Asn Lys Tyr Tyr Phe Asn Pro Asp Thr Ala Ile Ile  
 1970 1975 1980  
 Ser Lys Gly Trp Gln Thr Val Asn Gly Ser Arg Tyr Tyr Phe Asp Thr  
 1985 1990 1995 2000  
 15 Asp Thr Ala Ile Ala Phe Asn Gly Tyr Lys Thr Ile Asp Gly Lys His  
 2005 2010 2015  
 20 Phe Tyr Phe Asp Ser Asp Cys Val Val Lys Ile Gly Val Phe Ser Thr  
 2020 2025 2030  
 Ser Asn Gly Phe Glu Tyr Phe Ala Pro Ala Asn Thr Tyr Asn Asn Asn  
 2035 2040 2045  
 25 Ile Glu Gly Gln Ala Ile Val Tyr Gln Ser Lys Phe Leu Thr Leu Asn  
 2050 2055 2060  
 Gly Lys Lys Tyr Tyr Phe Asp Asn Asn Ser Lys Ala Val Thr Gly Leu  
 2065 2070 2075 2080  
 30 Gln Thr Ile Asp Ser Lys Lys Tyr Tyr Phe Asn Thr Asn Thr Ala Glu  
 2085 2090 2095  
 35 Ala Ala Thr Gly Trp Gln Thr Ile Asp Gly Lys Lys Tyr Tyr Phe Asn  
 2100 2105 2110  
 Thr Asn Thr Ala Glu Ala Ala Thr Gly Trp Gln Thr Ile Asp Gly Lys  
 2115 2120 2125  
 40 Lys Tyr Tyr Phe Asn Thr Asn Thr Ala Ile Ala Ser Thr Gly Tyr Thr  
 2130 2135 2140  
 Ile Ile Asn Gly Lys His Phe Tyr Phe Asn Thr Asp Gly Ile Met Gln  
 2145 2150 2155 2160  
 45 Ile Gly Val Phe Lys Gly Pro Asn Gly Phe Glu Tyr Phe Ala Pro Ala  
 2165 2170 2175  
 50 Asn Thr Asp Ala Asn Asn Ile Glu Gly Gln Ala Ile Leu Tyr Gln Asn  
 2180 2185 2190  
 Glu Phe Leu Thr Leu Asn Gly Lys Lys Tyr Tyr Phe Gly Ser Asp Ser  
 2195 2200 2205  
 55 Lys Ala Val Thr Gly Trp Arg Ile Ile Asn Asn Lys Lys Tyr Tyr Phe  
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 Asn Pro Asn Asn Ala Ile Ala Ala Ile His Leu Cys Thr Ile Asn Asn  
 2225 2230 2235 2240  
 60 Asp Lys Tyr Tyr Phe Ser Tyr Asp Gly Ile Leu Gln Asn Gly Tyr Ile  
 2245 2250 2255  
 65 Thr Ile Glu Arg Asn Asn Phe Tyr Phe Asp Ala Asn Asn Glu Ser Lys  
 2260 2265 2270  
 Met Val Thr Gly Val Phe Lys Gly Pro Asn Gly Phe Glu Tyr Phe Ala  
 2275 2280 2285

Pro Ala Asn Thr His Asn Asn Asn Ile Glu Gly Gln Ala Ile Val Tyr  
 2290 2295 2300  
 5 Gln Asn Lys Phe Leu Thr Leu Asn Gly Lys Lys Tyr Tyr Phe Asp Asn  
 2305 2310 2315 2320  
 Asp Ser Lys Ala Val Thr Gly Trp Gln Thr Ile Asp Gly Lys Lys Tyr  
 2325 2330 2335  
 10 Tyr Phe Asn Leu Asn Thr Ala Glu Ala Ala Thr Gly Trp Gln Thr Ile  
 2340 2345 2350  
 Asp Gly Lys Lys Tyr Tyr Phe Asn Leu Asn Thr Ala Glu Ala Ala Thr  
 2355 2360 2365  
 15 Gly Trp Gln Thr Ile Asp Gly Lys Lys Tyr Tyr Phe Asn Thr Asn Thr  
 2370 2375 2380  
 Phe Ile Ala Ser Thr Gly Tyr Thr Ser Ile Asn Gly Lys His Phe Tyr  
 2385 2390 2395 2400  
 Phe Asn Thr Asp Gly Ile Met Gln Ile Gly Val Phe Lys Gly Pro Asn  
 2405 2410 2415  
 25 Gly Phe Glu Tyr Phe Ala Pro Ala Asn Thr Asp Ala Asn Asn Ile Glu  
 2420 2425 2430  
 Gly Gln Ala Ile Leu Tyr Gln Asn Lys Phe Leu Thr Leu Asn Gly Lys  
 2435 2440 2445  
 30 Lys Tyr Tyr Phe Gly Ser Asp Ser Lys Ala Val Thr Gly Leu Arg Thr  
 2450 2455 2460  
 Ile Asp Gly Lys Lys Tyr Tyr Phe Asn Thr Asn Thr Ala Val Ala Val  
 2465 2470 2475 2480  
 Thr Gly Trp Gln Thr Ile Asn Gly Lys Lys Tyr Tyr Phe Asn Thr Asn  
 2485 2490 2495  
 40 Thr Ser Ile Ala Ser Thr Gly Tyr Thr Ile Ile Ser Gly Lys His Phe  
 2500 2505 2510  
 Tyr Phe Asn Thr Asp Gly Ile Met Gln Ile Gly Val Phe Lys Gly Pro  
 2515 2520 2525  
 45 Asp Gly Phe Glu Tyr Phe Ala Pro Ala Asn Thr Asp Ala Asn Asn Ile  
 2530 2535 2540  
 Glu Gly Gln Ala Ile Arg Tyr Gln Asn Arg Phe Leu Tyr Leu His Asp  
 2545 2550 2555 2560  
 Asn Ile Tyr Tyr Phe Gly Asn Asn Ser Lys Ala Ala Thr Gly Trp Val  
 2565 2570 2575  
 55 Thr Ile Asp Gly Asn Arg Tyr Tyr Phe Glu Pro Asn Thr Ala Met Gly  
 2580 2585 2590  
 Ala Asn Gly Tyr Lys Thr Ile Asp Asn Lys Asn Phe Tyr Phe Arg Asn  
 2595 2600 2605  
 60 Gly Leu Pro Gln Ile Gly Val Phe Lys Gly Ser Asn Gly Phe Glu Tyr  
 2610 2615 2620  
 Phe Ala Pro Ala Asn Thr Asp Ala Asn Asn Ile Glu Gly Gln Ala Ile  
 2625 2630 2635 2640  
 Arg Tyr Gln Asn Arg Phe Leu His Leu Leu Gly Lys Ile Tyr Tyr Phe  
 2645 2650 2655

Gly Asn Asn Ser Lys Ala Val Thr Gly Trp Gln Thr Ile Asn Gly Lys  
 2660 2665 2670  
 5 Val Tyr Tyr Phe Met Pro Asp Thr Ala Met Ala Ala Ala Gly Gly Leu  
 2675 2680 2685  
 Phe Glu Ile Asp Gly Val Ile Tyr Phe Phe Gly Val Asp Gly Val Lys  
 2690 2695 2700  
 10 Ala Pro Gly Ile Tyr Gly  
 2705 2710  
 (2) INFORMATION FOR SEQ ID NO:7:  
 15 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 811 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: unknown  
 (D) TOPOLOGY: unknown  
 20 (ii) MOLECULE TYPE: protein  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:  
 25 Ser Tyr Lys Ile Ile Asn Gly Lys His Phe Tyr Phe Asn Asn Asp Gly  
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 Val Met Gln Leu Gly Val Phe Lys Gly Pro Asp Gly Phe Glu Tyr Phe  
 20 25 30  
 30 Ala Pro Ala Asn Thr Gln Asn Asn Asn Ile Glu Gly Gln Ala Ile Val  
 35 40 45  
 Tyr Gln Ser Lys Phe Leu Thr Leu Asn Gly Lys Lys Tyr Tyr Phe Asp  
 50 55 60  
 Asn Asn Ser Lys Ala Val Thr Gly Trp Arg Ile Ile Asn Asn Glu Lys  
 65 70 75 80  
 40 Tyr Tyr Phe Asn Pro Asn Asn Ala Ile Ala Ala Val Gly Leu Gln Val  
 85 90 95  
 Ile Asp Asn Asn Lys Tyr Tyr Phe Asn Pro Asp Thr Ala Ile Ile Ser  
 100 105 110  
 45 Lys Gly Trp Gln Thr Val Asn Gly Ser Arg Tyr Tyr Phe Asp Thr Asp  
 115 120 125  
 Thr Ala Ile Ala Phe Asn Gly Tyr Lys Thr Ile Asp Gly Lys His Phe  
 130 135 140  
 50 Tyr Phe Asp Ser Asp Cys Val Val Lys Ile Gly Val Phe Ser Thr Ser  
 145 150 155 160  
 55 Asn Gly Phe Glu Tyr Phe Ala Pro Ala Asn Thr Tyr Asn Asn Asn Ile  
 165 170 175  
 Glu Gly Gln Ala Ile Val Tyr Gln Ser Lys Phe Leu Thr Leu Asn Gly  
 180 185 190  
 60 Lys Lys Tyr Tyr Phe Asp Asn Asn Ser Lys Ala Val Thr Gly Leu Gln  
 195 200 205  
 65 Thr Ile Asp Ser Lys Lys Tyr Tyr Phe Asn Thr Asn Thr Ala Glu Ala  
 210 215 220  
 Ala Thr Gly Trp Gln Thr Ile Asp Gly Lys Lys Tyr Tyr Phe Asn Thr  
 225 230 235 240

Asn Thr Ala Glu Ala Ala Thr Gly Trp Gln Thr Ile Asp Gly Lys Lys  
 245 250 255  
 5 Tyr Tyr Phe Asn Thr Asn Thr Ala Ile Ala Ser Thr Gly Tyr Thr Ile  
 260 265 270  
 Ile Asn Gly Lys His Phe Tyr Phe Asn Thr Asp Gly Ile Met Gln Ile  
 275 280 285  
 10 Gly Val Phe Lys Gly Pro Asn Gly Phe Glu Tyr Phe Ala Pro Ala Asn  
 290 295 300  
 15 Thr Asp Ala Asn Asn Ile Glu Gly Gln Ala Ile Leu Tyr Gln Asn Glu  
 305 310 315 320  
 Phe Leu Thr Leu Asn Gly Lys Lys Tyr Tyr Phe Gly Ser Asp Ser Lys  
 325 330 335  
 20 Ala Val Thr Gly Trp Arg Ile Ile Asn Asn Lys Lys Tyr Tyr Phe Asn  
 340 345 350  
 Pro Asn Asn Ala Ile Ala Ala Ile His Leu Cys Thr Ile Asn Asn Asp  
 355 360 365  
 25 Lys Tyr Tyr Phe Ser Tyr Asp Gly Ile Leu Gln Asn Gly Tyr Ile Thr  
 370 375 380  
 30 Ile Glu Arg Asn Asn Phe Tyr Phe Asp Ala Asn Asn Glu Ser Lys Met  
 385 390 395 400  
 Val Thr Gly Val Phe Lys Gly Pro Asn Gly Phe Glu Tyr Phe Ala Pro  
 405 410 415  
 35 Ala Asn Thr His Asn Asn Asn Ile Glu Gly Gln Ala Ile Val Tyr Gln  
 420 425 430  
 Asn Lys Phe Leu Thr Leu Asn Gly Lys Lys Tyr Tyr Phe Asp Asn Asp  
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 40 Ser Lys Ala Val Thr Gly Trp Gln Thr Ile Asp Gly Lys Lys Tyr Tyr  
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 45 Phe Asn Leu Asn Thr Ala Glu Ala Ala Thr Gly Trp Gln Thr Ile Asp  
 465 470 475 480  
 Gly Lys Lys Tyr Tyr Phe Asn Leu Asn Thr Ala Glu Ala Ala Thr Gly  
 485 490 495  
 50 Trp Gln Thr Ile Asp Gly Lys Lys Tyr Tyr Phe Asn Thr Asn Thr Phe  
 500 505 510  
 Ile Ala Ser Thr Gly Tyr Thr Ser Ile Asn Gly Lys His Phe Tyr Phe  
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 55 Asn Thr Asp Gly Ile Met Gln Ile Gly Val Phe Lys Gly Pro Asn Gly  
 530 535 540  
 60 Phe Glu Tyr Phe Ala Pro Ala Asn Thr Asp Ala Asn Asn Ile Glu Gly  
 545 550 555 560  
 Gln Ala Ile Leu Tyr Gln Asn Lys Phe Leu Thr Leu Asn Gly Lys Lys  
 565 570 575  
 65 Tyr Tyr Phe Gly Ser Asp Ser Lys Ala Val Thr Gly Leu Arg Thr Ile  
 580 585 590  
 Asp Gly Lys Lys Tyr Tyr Phe Asn Thr Asn Thr Ala Val Ala Val Thr  
 595 600 605

Gly Trp Gln Thr Ile Asn Gly Lys Lys Tyr Tyr Phe Asn Thr Asn Thr  
 610 615 620  
 5 Ser Ile Ala Ser Thr Gly Tyr Thr Ile Ile Ser Gly Lys His Phe Tyr  
 625 630 635 640  
 Phe Asn Thr Asp Gly Ile Met Gln Ile Gly Val Phe Lys Gly Pro Asp  
 645 650 655  
 10 Gly Phe Glu Tyr Phe Ala Pro Ala Asn Thr Asp Ala Asn Asn Ile Glu  
 660 665 670  
 Gly Gln Ala Ile Arg Tyr Gln Asn Arg Phe Leu Tyr Leu His Asp Asn  
 675 680 685  
 15 Ile Tyr Tyr Phe Gly Asn Asn Ser Lys Ala Ala Thr Gly Trp Val Thr  
 690 695 700  
 20 Ile Asp Gly Asn Arg Tyr Tyr Phe Glu Pro Asn Thr Ala Met Gly Ala  
 705 710 715 720  
 Asn Gly Tyr Lys Thr Ile Asp Asn Lys Asn Phe Tyr Phe Arg Asn Gly  
 725 730 735  
 25 Leu Pro Gln Ile Gly Val Phe Lys Gly Ser Asn Gly Phe Glu Tyr Phe  
 740 745 750  
 Ala Pro Ala Asn Thr Asp Ala Asn Asn Ile Glu Gly Gln Ala Ile Arg  
 755 760 765  
 30 Tyr Gln Asn Arg Phe Leu His Leu Leu Gly Lys Ile Tyr Tyr Phe Gly  
 770 775 780  
 35 Asn Asn Ser Lys Ala Val Thr Gly Trp Gln Thr Ile Asn Gly Lys Val  
 785 790 795 800  
 Tyr Tyr Phe Met Pro Asp Thr Ala Met Ala Ala  
 805 810

40 (2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 91 amino acids  
 45 (B) TYPE: amino acid  
 (C) STRANDEDNESS: unknown  
 (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

50 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Ser Tyr Lys Ile Ile Asn Gly Lys His Phe Tyr Phe Asn Asn Asp Gly  
 1 5 10 15  
 55 Val Met Gln Leu Gly Val Phe Lys Gly Pro Asp Gly Phe Glu Tyr Phe  
 20 25 30  
 Ala Pro Ala Asn Thr Gln Asn Asn Asn Ile Glu Gly Gln Ala Ile Val  
 35 40 45  
 60 Tyr Gln Ser Lys Phe Leu Thr Leu Asn Gly Lys Lys Tyr Tyr Phe Asp  
 50 55 60  
 65 Asn Asn Ser Lys Ala Val Thr Gly Trp Arg Ile Ile Asn Asn Glu Lys  
 65 70 75 80  
 Tyr Tyr Phe Asn Pro Asn Asn Ala Ile Ala Ala  
 85 90

## (2) INFORMATION FOR SEQ ID NO:9:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7101 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (ix) FEATURE:

- (A) NAME/KEY: CDS  
 (B) LOCATION: 1..7098

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

5	ATG AGT TTA GTT AAT AGA AAA CAG TTA GAA AAA ATG GCA AAT GTA AGA	48
	Met Ser Leu Val Asn Arg Lys Gln Leu Glu Lys Met Ala Asn Val Arg	
10	1 5 10 15	
20	TTT CGT ACT CAA GAA GAT GAA TAT GTT GCA ATA TTG GAT GCT TTA GAA	96
	Phe Arg Thr Gln Glu Asp Glu Tyr Val Ala Ile Leu Asp Ala Leu Glu	
	20 25 30	
25	GAA TAT CAT AAT ATG TCA GAG AAT ACT GTA GTC GAA AAA TAT TTA AAA	144
	Glu Tyr His Asn Met Ser Glu Asn Thr Val Val Glu Lys Tyr Leu Lys	
	35 40 45	
30	TTA AAA GAT ATA AAT AGT TTA ACA GAT ATT TAT ATA GAT ACA TAT AAA	192
	Leu Lys Asp Ile Asn Ser Leu Thr Asp Ile Tyr Ile Asp Thr Tyr Lys	
	50 55 60	
35	AAA TCT GGT AGA AAT AAA GCC TTA AAA AAA TTT AAG GAA TAT CTA GTT	240
	Lys Ser Gly Arg Asn Lys Ala Leu Lys Lys Phe Lys Glu Tyr Leu Val	
	65 70 75 80	
40	ACA GAA GTA TTA GAG CTA AAG AAT AAT AAT TTA ACT CCA GTT GAG AAA	288
	Thr Glu Val Leu Glu Leu Lys Asn Asn Asn Leu Thr Pro Val Glu Lys	
	85 90 95	
45	AAT TTA CAT TTT GTT TGG ATT GGA GGT CAA ATA AAT GAC ACT GCT ATT	336
	Asn Leu His Phe Val Trp Ile Gly Gly Gln Ile Asn Asp Thr Ala Ile	
	100 105 110	
50	AAT TAT ATA AAT CAA TGG AAA GAT GTA AAT AGT GAT TAT AAT GTT AAT	384
	Asn Tyr Ile Asn Gln Trp Lys Asp Val Asn Ser Asp Tyr Asn Val Asn	
	115 120 125	
55	GTT TTT TAT GAT AGT AAT GCA TTT TTG ATA AAC ACA TTG AAA AAA ACT	432
	Val Phe Tyr Asp Ser Asn Ala Phe Leu Ile Asn Thr Leu Lys Lys Thr	
	130 135 140	
60	GTA GTA GAA TCA GCA ATA AAT GAT ACA CTT GAA TCA TTT AGA GAA AAC	480
	Val Val Glu Ser Ala Ile Asn Asp Thr Leu Glu Ser Phe Arg Glu Asn	
	145 150 155 160	
65	TTA AAT GAC CCT AGA TTT GAC TAT AAT AAA TTC TTC AGA AAA CGT ATG	528
	Leu Asn Asp Pro Arg Phe Asp Tyr Asn Lys Phe Phe Arg Lys Arg Met	
	165 170 175	
70	GAA ATA ATT TAT GAT AAA CAG AAA AAT TTC ATA AAC TAC TAT AAA GCT	576
	Glu Ile Ile Tyr Asp Lys Gln Lys Asn Phe Ile Asn Tyr Tyr Lys Ala	
	180 185 190	
75	CAA AGA GAA GAA AAT CCT GAA CTT ATA ATT GAT GAT ATT GTA AAG ACA	624
	Gln Arg Glu Glu Asn Pro Glu Leu Ile Ile Asp Asp Ile Val Lys Thr	
	195 200 205	

	TAT CTT TCA AAT GAG TAT TCA AAG GAG ATA GAT GAA CTT AAT ACC TAT Tyr Leu Ser Asn Glu Tyr Ser Lys Glu Ile Asp Glu Leu Asn Thr Tyr	672
5	ATT GAA GAA TCC TTA AAT AAA ATT ACA CAG AAT AGT GGA AAT GAT GTT Ile Glu Glu Ser Leu Asn Lys Ile Thr Gln Asn Ser Gly Asn Asp Val	720
10	AGA AAC TTT GAA GAA TTT AAA AAT GGA GAG TCA TTC AAC TTA TAT GAA Arg Asn Phe Glu Glu Phe Lys Asn Gly Glu Ser Phe Asn Leu Tyr Glu	768
15	CAA GAG TTG GTA GAA AGG TGG AAT TTA GCT GCT GCT TCT GAC ATA TTA Gln Glu Leu Val Glu Arg Trp Asn Leu Ala Ala Ser Asp Ile Leu	816
20	AGA ATA TCT GCA TTA AAA GAA ATT GGT GGT ATG TAT TTA GAT GTT GAT Arg Ile Ser Ala Leu Lys Glu Ile Gly Gly Met Tyr Leu Asp Val Asp	864
25	ATG TTA CCA GGA ATA CAA CCA GAC TTA TTT GAG TCT ATA GAG AAA CCT Met Leu Pro Gly Ile Gln Pro Asp Leu Phe Glu Ser Ile Glu Lys Pro	912
30	AGT TCA GTA ACA GTG GAT TTT TGG GAA ATG ACA AAG TTA GAA GCT ATA Ser Ser Val Thr Val Asp Phe Trp Glu Met Thr Lys Leu Glu Ala Ile	960
35	ATG AAA TAC AAA GAA TAT ATA CCA GAA TAT ACC TCA GAA CAT TTT GAC Met Lys Tyr Lys Glu Tyr Ile Pro Glu Tyr Thr Ser Glu His Phe Asp	1008
40	ATG TTA GAC GAA GAA GTT CAA AGT AGT TTT GAA TCT GTT CTA GCT TCT Met Leu Asp Glu Glu Val Gln Ser Ser Phe Glu Ser Val Leu Ala Ser	1056
45	AAG TCA GAT AAA TCA GAA ATA TTC TCA TCA CTT GGT GAT ATG GAG GCA Lys Ser Asp Lys Ser Glu Ile Phe Ser Ser Leu Gly Asp Met Glu Ala	1104
50	TCA CCA CTA GAA GTT AAA ATT GCA TTT AAT AGT AAG GGT ATT ATA AAT Ser Pro Leu Glu Val Lys Ile Ala Phe Asn Ser Lys Gly Ile Ile Asn	1152
55	CAA GGG CTA ATT TCT GTG AAA GAC TCA TAT TGT AGC AAT TTA ATA GTA Gln Gly Leu Ile Ser Val Lys Asp Ser Tyr Cys Ser Asn Leu Ile Val	1200
60	AAA CAA ATC GAG AAT AGA TAT AAA ATA TTG AAT AAT AGT TTA AAT CCA Lys Gln Ile Glu Asn Arg Tyr Lys Ile Leu Asn Asn Ser Leu Asn Pro	1248
65	GCT ATT AGC GAG GAT AAT GAT TTT AAT ACT ACA ACG AAT ACC TTT ATT Ala Ile Ser Glu Asp Asn Asp Phe Asn Thr Thr Thr Asn Thr Phe Ile	1296
70	GAT AGT ATA ATG GCT GAA GCT AAT GCA GAT AAT GGT AGA TTT ATG ATG Asp Ser Ile Met Ala Glu Ala Asn Ala Asp Asn Gly Arg Phe Met Met	1344
75	GAA CTA GGA AAG TAT TTA AGA GTT GGT TTC TTC CCA GAT GTT AAA ACT Glu Leu Gly Lys Tyr Leu Arg Val Gly Phe Phe Pro Asp Val Lys Thr	1392
80	ACT ATT AAC TTA AGT GGC CCT GAA GCA TAT GCG GCA GCT TAT CAA GAT Thr Ile Asn Leu Ser Gly Pro Glu Ala Tyr Ala Ala Ala Tyr Gln Asp	1440



	TTA TTA ATG TTT AAA GAA GGC AGT ATG AAT ATC CAT TTG ATA GAA GCT Leu Leu Met Phe Lys Glu Gly Ser Met Asn Ile His Leu Ile Glu Ala	1488
	485 490 495	
5	GAT TTA AGA AAC TTT GAA ATC TCT AAA ACT AAT ATT TCT CAA TCA ACT Asp Leu Arg Asn Phe Glu Ile Ser Lys Thr Asn Ile Ser Gln Ser Thr	1536
	500 505 510	
10	GAA CAA GAA ATG GCT AGC TTA TGG TCA TTT GAC GAT GCA AGA GCT AAA Glu Gln Glu Met Ala Ser Leu Trp Ser Phe Asp Asp Ala Arg Ala Lys	1584
	515 520 525	
15	GCT CAA TTT GAA GAA TAT AAA AGG AAT TAT TTT GAA GGT TCT CTT GGT Ala Gln Phe Glu Glu Tyr Lys Arg Asn Tyr Phe Glu Gly Ser Leu Gly	1632
	530 535 540	
20	GAA GAT GAT AAT CTT GAT TTT TCT CAA AAT ATA GTA GTT GAC AAG GAG Glu Asp Asp Asn Leu Asp Phe Ser Gln Asn Ile Val Val Asp Lys Glu	1680
	545 550 555 560	
	TAT CTT TTA GAA AAA ATA TCT TCA TTA GCA AGA AGT TCA GAG AGA GGA Tyr Leu Leu Glu Lys Ile Ser Ser Leu Ala Arg Ser Ser Glu Arg Gly	1728
	565 570 575	
25	TAT ATA CAC TAT ATT GTT CAG TTA CAA GGA GAT AAA ATT AGT TAT GAA Tyr Ile His Tyr Ile Val Gln Leu Gln Gly Asp Lys Ile Ser Tyr Glu	1776
	580 585 590	
30	GCA GCA TGT AAC TTA TTT GCA AAG ACT CCT TAT GAT AGT GTA CTG TTT Ala Ala Cys Asn Leu Phe Ala Lys Thr Pro Tyr Asp Ser Val Leu Phe	1824
	595 600 605	
35	CAG AAA AAT ATA GAA GAT TCA GAA ATT GCA TAT TAT TAT AAT CCT GGA Gln Lys Asn Ile Glu Asp Ser Glu Ile Ala Tyr Tyr Tyr Asn Pro Gly	1872
	610 615 620	
40	GAT GGT GAA ATA CAA GAA ATA GAC AAG TAT AAA ATT CCA AGT ATA ATT Asp Gly Glu Ile Gln Glu Ile Asp Lys Tyr Lys Ile Pro Ser Ile Ile	1920
	625 630 635 640	
	TCT GAT AGA CCT AAG ATT AAA TTA ACA TTT ATT GGT CAT GGT AAA GAT Ser Asp Arg Pro Lys Ile Lys Leu Thr Phe Ile Gly His Gly Lys Asp	1968
	645 650 655	
45	GAA TTT AAT ACT GAT ATA TTT GCA GGT TTT GAT GTA GAT TCA TTA TCC Glu Phe Asn Thr Asp Ile Phe Ala Gly Phe Asp Val Asp Ser Leu Ser	2016
	660 665 670	
50	ACA GAA ATA GAA GCA GCA ATA GAT TTA GCT AAA GAG GAT ATT TCT CCT Thr Glu Ile Glu Ala Ala Ile Asp Leu Ala Lys Glu Asp Ile Ser Pro	2064
	675 680 685	
55	AAG TCA ATA GAA ATA AAT TTA TTA GGA TGT AAT ATG TTT AGC TAC TCT Lys Ser Ile Glu Ile Asn Leu Leu Gly Cys Asn Met Phe Ser Tyr Ser	2112
	690 695 700	
60	ATC AAC GTA GAG GAG ACT TAT CCT GGA AAA TTA TTA CTT AAA GTT AAA Ile Asn Val Glu Glu Thr Tyr Pro Gly Lys Leu Leu Leu Lys Val Lys	2160
	705 710 715 720	
	GAT AAA ATA TCA GAA TTA ATG CCA TCT ATA AGT CAA GAC TCT ATT ATA Asp Lys Ile Ser Glu Leu Met Pro Ser Ile Ser Gln Asp Ser Ile Ile	2208
	725 730 735	
65	GTA AGT GCA AAT CAA TAT GAA GTT AGA ATA AAT AGT GAA GGA AGA AGA Val Ser Ala Asn Gln Tyr Glu Val Arg Ile Asn Ser Glu Gly Arg Arg	2256
	740 745 750	

	GAA TTA TTG GAT CAT TCT GGT GAA TGG ATA AAT AAA GAA GAA AGT ATT Glu Leu Leu Asp His Ser Gly Glu Trp Ile Asn Lys Glu Glu Ser Ile	2304
5	ATA AAG GAT ATT TCA TCA AAA GAA TAT ATA TCA TTT AAT CCT AAA GAA Ile Lys Asp Ile Ser Ser Lys Glu Tyr Ile Ser Phe Asn Pro Lys Glu	2352
10	AAT AAA ATT ACA GTA AAA TCT AAA AAT TTA CCT GAG CTA TCT ACA TTA Asn Lys Ile Thr Val Lys Ser Lys Asn Leu Pro Glu Leu Ser Thr Leu	2400
15	TTA CAA GAA ATT AGA AAT AAT TCT AAT TCA AGT GAT ATT GAA CTA GAA Leu Gln Glu Ile Arg Asn Asn Ser Asn Ser Asp Ile Glu Ser Glu	2448
20	GAA AAA GTA ATG TTA ACA GAA TGT GAG ATA AAT GTT ATT TCA AAT ATA Glu Lys Val Met Leu Thr Glu Cys Glu Ile Asn Val Ile Ser Asn Ile	2496
25	GAT ACG CAA ATT GTT GAG GAA AGG ATT GAA GAA GCT AAG AAT TTA ACT Asp Thr Gln Ile Val Glu Glu Arg Ile Glu Glu Ala Lys Asn Leu Thr	2544
30	TCT GAC TCT ATT AAT TAT ATA AAA GAT GAA TTT AAA CTA ATA GAA TCT Ser Asp Ser Ile Asn Tyr Ile Lys Asp Glu Phe Lys Leu Ile Glu Ser	2592
35	ATT TCT GAT GCA CTA TGT GAC TTA AAA CAA CAG AAT GAA TTA GAA GAT Ile Ser Asp Ala Leu Cys Asp Leu Lys Gln Gln Asn Glu Leu Glu Asp	2640
40	TCT CAT TTT ATA TCT TTT GAG GAC ATA TCA GAG ACT GAT GAG GGA TTT Ser His Phe Ile Ser Phe Glu Asp Ile Ser Glu Thr Asp Glu Gly Phe	2688
45	AGT ATA AGA TTT ATT AAT AAA GAA ACT GGA GAA TCT ATA TTT GTA GAA Ser Ile Arg Phe Ile Asn Lys Glu Thr Gly Glu Ser Ile Phe Val Glu	2736
50	ACT GAA AAA ACA ATA TTC TCT GAA TAT GCT AAT CAT ATA ACT GAA GAG Thr Glu Lys Thr Ile Phe Ser Glu Tyr Ala Asn His Ile Thr Glu Glu	2784
55	ATT TCT AAG ATA AAA GGT ACT ATA TTT GAT ACT GTA AAT GGT AAG TTA Ile Ser Lys Ile Lys Gly Thr Ile Phe Asp Thr Val Asn Gly Lys Leu	2832
60	GTA AAA AAA GTA AAT TTA GAT ACT ACA CAC GAA GTA AAT ACT TTA AAT Val Lys Lys Val Asn Leu Asp Thr Thr His Glu Val Asn Thr Leu Asn	2880
	GCT GCA TTT TTT ATA CAA TCA TTA ATA GAA TAT AAT AGT TCT AAA GAA Ala Ala Phe Phe Ile Gln Ser Leu Ile Glu Tyr Asn Ser Ser Lys Glu	2928
	TCT CTT AGT AAT TTA AGT GTA GCA ATG AAA GTC CAA GTT TAC GCT CAA Ser Leu Ser Asn Leu Ser Val Ala Met Lys Val Gln Val Tyr Ala Gln	2976
	TTA TTT AGT ACT GGT TTA AAT ACT ATT ACA GAT GCA GCC AAA GTT GTT Leu Phe Ser Thr Gly Leu Asn Thr Ile Thr Asp Ala Ala Lys Val Val	3024

	GAA TTA GTA TCA ACT GCA TTA GAT GAA ACT ATA GAC TTA CTT CCT ACA Glu Leu Val Ser Thr Ala Leu Asp Glu Thr Ile Asp Leu Leu Pro Thr 1010 1015 1020	3072
5	TTA TCT GAA GGA TTA CCT ATA ATT GCA ACT ATT ATA GAT GGT GTA AGT Leu Ser Glu Gly Leu Pro Ile Ile Ala Thr Ile Ile Asp Gly Val Ser 1025 1030 1035 1040	3120
10	TTA GGT GCA GCA ATC AAA GAG CTA AGT GAA ACG AGT GAC CCA TTA TTA Leu Gly Ala Ala Ile Lys Glu Leu Ser Glu Thr Ser Asp Pro Leu Leu 1045 1050 1055	3168
15	AGA CAA GAA ATA GAA GCT AAG ATA GGT ATA ATG GCA GTA AAT TTA ACA Arg Gln Glu Ile Glu Ala Lys Ile Gly Ile Met Ala Val Asn Leu Thr 1060 1065 1070	3216
20	ACA GCT ACA ACT GCA ATC ATT ACT TCA TCT TTG GGG ATA GCT AGT GGA Thr Ala Thr Thr Ala Ile Ile Thr Ser Ser Leu Gly Ile Ala Ser Gly 1075 1080 1085	3264
	TTT AGT ATA CTT TTA GTT CCT TTA GCA GGA ATT TCA GCA GGT ATA CCA Phe Ser Ile Leu Leu Val Pro Leu Ala Gly Ile Ser Ala Gly Ile Pro 1090 1095 1100	3312
25	AGC TTA GTA AAC AAT GAA CTT GTA CTT CGA GAT AAG GCA ACA AAG GTT Ser Leu Val Asn Asn Glu Leu Val Leu Arg Asp Lys Ala Thr Lys Val 1105 1110 1115 1120	3360
30	GTA GAT TAT TTT AAA CAT GTT TCA TTA GTT GAA ACT GAA GGA GTA TTT Val Asp Tyr Phe Lys His Val Ser Leu Val Glu Thr Glu Gly Val Phe 1125 1130 1135	3408
35	ACT TTA TTA GAT GAT AAA ATA ATG ATG CCA CAA GAT GAT TTA GTG ATA Thr Leu Leu Asp Asp Lys Ile Met Met Pro Gln Asp Asp Leu Val Ile 1140 1145 1150	3456
40	TCA GAA ATA GAT TTT AAT AAT AAT TCA ATA GTT TTA GGT AAA TGT GAA Ser Glu Ile Asp Phe Asn Asn Asn Ser Ile Val Leu Gly Lys Cys Glu 1155 1160 1165	3504
	ATC TGG AGA ATG GAA GGT GGT TCA GGT CAT ACT GTA ACT GAT GAT ATA Ile Trp Arg Met Glu Gly Ser Gly His Thr Val Thr Asp Asp Ile 1170 1175 1180	3552
45	GAT CAC TTC TTT TCA GCA CCA TCA ATA ACA TAT AGA GAG CCA CAC TTA Asp His Phe Phe Ser Ala Pro Ser Ile Thr Tyr Arg Glu Pro His Leu 1185 1190 1195 1200	3600
50	TCT ATA TAT GAC GTA TTG GAA GTA CAA AAA GAA GAA CTT GAT TTG TCA Ser Ile Tyr Asp Val Leu Glu Val Gln Lys Glu Glu Leu Asp Leu Ser 1205 1210 1215	3648
55	AAA GAT TTA ATG GTA TTA CCT AAT GCT CCA AAT AGA GTA TTT GCT TGG Lys Asp Leu Met Val Leu Pro Asn Ala Pro Asn Arg Val Phe Ala Trp 1220 1225 1230	3696
60	GAA ACA GGA TGG ACA CCA GGT TTA AGA AGC TTA GAA AAT GAT GGC ACA Glu Thr Gly Trp Thr Pro Gly Leu Arg Ser Leu Glu Asn Asp Gly Thr 1235 1240 1245	3744
	AAA CTG TTA GAC CGT ATA AGA GAT AAC TAT GAA GGT GAG TTT TAT TGG Lys Leu Leu Asp Arg Ile Arg Asp Asn Tyr Glu Gly Glu Phe Tyr Trp 1250 1255 1260	3792
65	AGA TAT TTT GCT TTT ATA GCT GAT GCT TTA ATA ACA ACA TTA AAA CCA Arg Tyr Phe Ala Phe Ile Ala Asp Ala Leu Ile Thr Thr Leu Lys Pro 1265 1270 1275 1280	3840

	AGA TAT GAA GAT ACT AAT ATA AGA ATA AAT TTA GAT AGT AAT ACT AGA Arg Tyr Glu Asp Thr Asn Ile Arg Ile Asn Leu Asp Ser Asn Thr Arg 1285 1290 1295	3888
5	AGT TTT ATA GTT CCA ATA ATA ACT ACA GAA TAT ATA AGA GAA AAA TTA Ser Phe Ile Val Pro Ile Ile Thr Thr Glu Tyr Ile Arg Glu Lys Leu 1300 1305 1310	3936
10	TCA TAT TCT TTC TAT GGT TCA GGA GGA ACT TAT GCA TTG TCT CTT TCT Ser Tyr Ser Phe Tyr Gly Ser Gly Gly Thr Tyr Ala Leu Ser Leu Ser 1315 1320 1325	3984
15	CAA TAT AAT ATG GGT ATA AAT ATA GAA TTA AGT GAA AGT GAT GTT TGG Gln Tyr Asn Met Gly Ile Asn Ile Glu Leu Ser Glu Ser Asp Val Trp 1330 1335 1340	4032
20	ATT ATA GAT GTT GAT AAT GTT GTG AGA GAT GTA ACT ATA GAA TCT GAT Ile Ile Asp Val Asp Asn Val Val Arg Asp Val Thr Ile Glu Ser Asp 1345 1350 1355 1360	4080
	AAA ATT AAA AAA GGT GAT TTA ATA GAA GGT ATT TTA TCT ACA CTA AGT Lys Ile Lys Lys Gly Asp Leu Ile Glu Gly Ile Leu Ser Thr Leu Ser 1365 1370 1375	4128
25	ATT GAA GAG AAT AAA ATT ATC TTA AAT AGC CAT GAG ATT AAT TTT TCT Ile Glu Glu Asn Lys Ile Ile Leu Asn Ser His Glu Ile Asn Phe Ser 1380 1385 1390	4176
30	GGT GAG GTA AAT GGA AGT AAT GGA TTT GTT TCT TTA ACA TTT TCA ATT Gly Glu Val Asn Gly Ser Asn Gly Phe Val Ser Leu Thr Phe Ser Ile 1395 1400 1405	4224
35	TTA GAA GGA ATA AAT GCA ATT ATA GAA GTT GAT TTA TTA TCT AAA TCA Leu Glu Gly Ile Asn Ala Ile Ile Glu Val Asp Leu Leu Ser Lys Ser 1410 1415 1420	4272
40	TAT AAA TTA CTT ATT TCT GGC GAA TTA AAA ATA TTG ATG TTA AAT TCA Tyr Lys Leu Leu Ile Ser Gly Glu Leu Lys Ile Leu Met Leu Asn Ser 1425 1430 1435 1440	4320
	AAT CAT ATT CAA CAG AAA ATA GAT TAT ATA GGA TTC AAT AGC GAA TTA Asn His Ile Gln Gln Lys Ile Asp Tyr Ile Gly Phe Asn Ser Glu Leu 1445 1450 1455	4368
45	CAG AAA AAT ATA CCA TAT AGC TTT GTA GAT AGT GAA GGA AAA GAG AAT Gln Lys Asn Ile Pro Tyr Ser Phe Val Asp Ser Glu Gly Lys Glu Asn 1460 1465 1470	4416
50	GGT TTT ATT AAT GGT TCA ACA AAA GAA GGT TTA TTT GTA TCT GAA TTA Gly Phe Ile Asn Gly Ser Thr Lys Glu Gly Leu Phe Val Ser Glu Leu 1475 1480 1485	4464
55	CCT GAT GTA GTT CTT ATA AGT AAG GTT TAT ATG GAT GAT AGT AAG CCT Pro Asp Val Val Leu Ile Ser Lys Val Tyr Met Asp Asp Ser Lys Pro 1490 1495 1500	4512
60	TCA TTT GGA TAT TAT AGT AAT AAT TTG AAA GAT GTC AAA GTT ATA ACT Ser Phe Gly Tyr Tyr Ser Asn Asn Leu Lys Asp Val Lys Val Ile Thr 1505 1510 1515 1520	4560
	AAA GAT AAT GTT AAT ATA TTA ACA GGT TAT TAT CTT AAG GAT GAT ATA Lys Asp Asn Val Asn Ile Leu Thr Gly Tyr Tyr Leu Lys Asp Asp Ile 1525 1530 1535	4608
65	AAA ATC TCT CTT TCT TTG ACT CTA CAA GAT GAA AAA ACT ATA AAG TTA Lys Ile Ser Leu Ser Leu Thr Leu Gln Asp Glu Lys Thr Ile Lys Leu 1540 1545 1550	4656

	AAT AGT GTG CAT TTA GAT GAA AGT GGA GTA GCT GAG ATT TTG AAG TTC Asn Ser Val His Leu Asp Glu Ser Gly Val Ala Glu Ile Leu Lys Phe 1555 1560 1565	4704
5	ATG AAT AGA AAA GGT AAT ACA AAT ACT TCA GAT TCT TTA ATG AGC TTT Met Asn Arg Lys Gly Asn Thr Asn Thr Ser Asp Ser Leu Met Ser Phe 1570 1575 1580	4752
10	TTA GAA AGT ATG AAT ATA AAA AGT ATT TTC GTT AAT TTC TTA CAA TCT Leu Glu Ser Met Asn Ile Lys Ser Ile Phe Val Asn Phe Leu Gln Ser 1585 1590 1595 1600	4800
15	AAT ATT AAG TTT ATA TTA GAT GCT AAT TTT ATA ATA AGT GGT ACT ACT Asn Ile Lys Phe Ile Leu Asp Ala Asn Phe Ile Ile Ser Gly Thr Thr 1605 1610 1615	4848
20	TCT ATT GGC CAA TTT GAG TTT ATT TGT GAT GAA AAT GAT AAT ATA CAA Ser Ile Gly Gln Phe Glu Phe Ile Cys Asp Glu Asn Asp Asn Ile Gln 1620 1625 1630	4896
	CCA TAT TTC ATT AAG TTT AAT ACA CTA GAA ACT AAT TAT ACT TTA TAT Pro Tyr Phe Ile Lys Phe Asn Thr Leu Glu Thr Asn Tyr Thr Leu Tyr 1635 1640 1645	4944
25	GTA GGA AAT AGA CAA AAT ATG ATA GTG GAA CCA AAT TAT GAT TTA GAT Val Gly Asn Arg Gln Asn Met Ile Val Glu Pro Asn Tyr Asp Leu Asp 1650 1655 1660	4992
30	GAT TCT GGA GAT ATA TCT TCA ACT GTT ATC AAT TTC TCT CAA AAG TAT Asp Ser Gly Asp Ile Ser Ser Thr Val Ile Asn Phe Ser Gln Lys Tyr 1665 1670 1675 1680	5040
35	CTT TAT GGA ATA GAC AGT TGT GTT AAT AAA GTT GTA ATT TCA CCA AAT Leu Tyr Gly Ile Asp Ser Cys Val Asn Lys Val Val Ile Ser Pro Asn 1685 1690 1695	5088
40	ATT TAT ACA GAT GAA ATA AAT ATA ACG CCT GTA TAT GAA ACA AAT AAT Ile Tyr Thr Asp Glu Ile Asn Ile Thr Pro Val Tyr Glu Thr Asn Asn 1700 1705 1710	5136
	ACT TAT CCA GAA GTT ATT GTA TTA GAT GCA AAT TAT ATA AAT GAA AAA Thr Tyr Pro Glu Val Ile Val Leu Asp Ala Asn Tyr Ile Asn Glu Lys 1715 1720 1725	5184
45	ATA AAT GTT AAT ATC AAT GAT CTA TCT ATA CGA TAT GTA TGG AGT AAT Ile Asn Val Asn Ile Asn Asp Leu Ser Ile Arg Tyr Val Trp Ser Asn 1730 1735 1740	5232
50	GAT GGT AAT GAT TTT ATT CTT ATG TCA ACT AGT GAA GAA AAT AAG GTG Asp Gly Asn Asp Phe Ile Leu Met Ser Thr Ser Glu Glu Asn Lys Val 1745 1750 1755 1760	5280
55	TCA CAA GTT AAA ATA AGA TTC GTT AAT GTT TTT AAA GAT AAG ACT TTG Ser Gln Val Lys Ile Arg Phe Val Asn Val Phe Lys Asp Lys Thr Leu 1765 1770 1775	5328
60	GCA AAT AAG CTA TCT TTT AAC TTT AGT GAT AAA CAA GAT GTA CCT GTA Ala Asn Lys Leu Ser Phe Asn Phe Ser Asp Lys Gln Asp Val Pro Val 1780 1785 1790	5376
	AGT GAA ATA ATC TTA TCA TTT ACA CCT TCA TAT TAT GAG GAT GGA TTG Ser Glu Ile Ile Leu Ser Phe Thr Pro Ser Tyr Tyr Glu Asp Gly Leu 1795 1800 1805	5424
65	ATT GGC TAT GAT TTG GGT CTA GTT TCT TTA TAT AAT GAG AAA TTT TAT Ile Gly Tyr Asp Leu Gly Leu Val Ser Leu Tyr Asn Glu Lys Phe Tyr 1810 1815 1820	5472

	ATT AAT AAC TTT GGA ATG ATG GTA TCT GGA TTA ATA TAT ATT AAT GAT Ile Asn Asn Phe Gly Met Met Val Ser Gly Leu Ile Tyr Ile Asn Asp 1825 1830 1835 1840	5520
5	TCA TTA TAT TAT TTT AAA CCA CCA GTA AAT AAT TTG ATA ACT GGA TTT Ser Leu Tyr Tyr Phe Lys Pro Pro Val Asn Asn Leu Ile Thr Gly Phe 1845 1850 1855	5568
10	GTG ACT GTA GGC GAT GAT AAA TAC TAC TTT AAT CCA ATT AAT GGT GGA Val Thr Val Gly Asp Asp Lys Tyr Tyr Phe Asn Pro Ile Asn Gly Gly 1860 1865 1870	5616
15	GCT GCT TCA ATT GGA GAG ACA ATA ATT GAT GAC AAA AAT TAT TAT TTC Ala Ala Ser Ile Gly Glu Thr Ile Ile Asp Asp Lys Asn Tyr Tyr Phe 1875 1880 1885	5664
20	AAC CAA AGT GGA GTG TTA CAA ACA GGT GTA TTT AGT ACA GAA GAT GGA Asn Gln Ser Gly Val Leu Gln Thr Gly Val Phe Ser Thr Glu Asp Gly 1890 1895 1900	5712
	TTT AAA TAT TTT GCC CCA GCT AAT ACA CTT GAT GAA AAC CTA GAA GGA Phe Lys Tyr Phe Ala Pro Ala Asn Thr Leu Asp Glu Asn Leu Glu Gly 1905 1910 1915 1920	5760
25	GAA GCA ATT GAT TTT ACT GGA AAA TTA ATT ATT GAC GAA AAT ATT TAT Glu Ala Ile Asp Phe Thr Gly Lys Leu Ile Ile Asp Glu Asn Ile Tyr 1925 1930 1935	5808
30	TAT TTT GAT GAT AAT TAT AGA GGA GCT GTA GAA TGG AAA GAA TTA GAT Tyr Phe Asp Asp Asn Tyr Arg Gly Ala Val Glu Trp Lys Glu Leu Asp 1940 1945 1950	5856
35	GGT GAA ATG CAC TAT TTT AGC CCA GAA ACA GGT AAA GCT TTT AAA GGT Gly Glu Met His Tyr Phe Ser Pro Glu Thr Gly Lys Ala Phe Lys Gly 1955 1960 1965	5904
40	CTA AAT CAA ATA GGT GAT TAT AAA TAC TAT TTC AAT TCT GAT GGA GTT Leu Asn Gln Ile Gly Asp Tyr Lys Tyr Tyr Phe Asn Ser Asp Gly Val 1970 1975 1980	5952
	ATG CAA AAA GGA TTT GTT AGT ATA AAT GAT AAT AAA CAC TAT TTT GAT Met Gln Lys Gly Phe Val Ser Ile Asn Asp Asn Lys His Tyr Phe Asp 1985 1990 1995 2000	6000
45	GAT TCT GGT GTT ATG AAA GTA GGT TAC ACT GAA ATA GAT GGC AAG CAT Asp Ser Gly Val Met Lys Val Gly Tyr Thr Glu Ile Asp Gly Lys His 2005 2010 2015	6048
50	TTC TAC TTT GCT GAA AAC GGA GAA ATG CAA ATA GGA GTA TTT AAT ACA Phe Tyr Phe Ala Glu Asn Gly Glu Met Gln Ile Gly Val Phe Asn Thr 2020 2025 2030	6096
55	GAA GAT GGA TTT AAA TAT TTT GCT CAT CAT AAT GAA GAT TTA GGA AAT Glu Asp Gly Phe Lys Tyr Phe Ala His His Asn Glu Asp Leu Gly Asn 2035 2040 2045	6144
60	GAA GAA GGT GAA GAA ATC TCA TAT TCT GGT ATA TTA AAT TTC AAT AAT Glu Glu Gly Glu Glu Ile Ser Tyr Ser Gly Ile Leu Asn Phe Asn Asn 2050 2055 2060	6192
	AAA ATT TAC TAT TTT GAT GAT TCA TTT ACA GCT GTA GTT GGA TGG AAA Lys Ile Tyr Tyr Phe Asp Asp Ser Phe Thr Ala Val Val Gly Trp Lys 2065 2070 2075 2080	6240
65	GAT TTA GAG GAT GGT TCA AAG TAT TAT TTT GAT GAA GAT ACA GCA GAA Asp Leu Glu Asp Gly Ser Lys Tyr Tyr Phe Asp Glu Asp Thr Ala Glu 2085 2090 2095	6288

	GCA TAT ATA GGT TTG TCA TTA ATA AAT GAT GGT CAA TAT TAT TTT AAT Ala Tyr Ile Gly Leu Ser Leu Ile Asn Asp Gly Gln Tyr Tyr Phe Asn 2100 2105 2110	6336
5	GAT GAT GGA ATT ATG CAA GTT GGA TTT GTC ACT ATA AAT GAT AAA GTC Asp Asp Gly Ile Met Gln Val Gly Phe Val Thr Ile Asn Asp Lys Val 2115 2120 2125	6384
10	TTC TAC TTC TCT GAC TCT GGA ATT ATA GAA TCT GGA GTA CAA AAC ATA Phe Tyr Phe Ser Asp Ser Gly Ile Ile Glu Ser Gly Val Gln Asn Ile 2130 2135 2140	6432
15	GAT GAC AAT TAT TTC TAT ATA GAT GAT AAT GGT ATA GTT CAA ATT GGT Asp Asp Asn Tyr Phe Tyr Ile Asp Asp Asn Gly Ile Val Gln Ile Gly 2145 2150 2155 2160	6480
20	GTA TTT GAT ACT TCA GAT GGA TAT AAA TAT TTT GCA CCT GCT AAT ACT Val Phe Asp Thr Ser Asp Gly Tyr Lys Tyr Phe Ala Pro Ala Asn Thr 2165 2170 2175	6528
	GTA AAT GAT AAT ATT TAC GGA CAA GCA GTT GAA TAT AGT GGT TTA GTT Val Asn Asp Asn Ile Tyr Gly Gln Ala Val Glu Tyr Ser Gly Leu Val 2180 2185 2190	6576
25	AGA GTT GGG GAA GAT GTA TAT TAT TTT GGA GAA ACA TAT ACA ATT GAG Arg Val Gly Glu Asp Val Tyr Tyr Phe Gly Glu Thr Tyr Thr Ile Glu 2195 2200 2205	6624
30	ACT GGA TGG ATA TAT GAT ATG GAA AAT GAA AGT GAT AAA TAT TAT TTC Thr Gly Trp Ile Tyr Asp Met Glu Asn Glu Ser Asp Lys Tyr Tyr Phe 2210 2215 2220	6672
35	AAT CCA GAA ACT AAA AAA GCA TGC AAA GGT ATT AAT TTA ATT GAT GAT Asn Pro Glu Thr Lys Lys Ala Cys Lys Gly Ile Asn Leu Ile Asp Asp 2225 2230 2235 2240	6720
40	ATA AAA TAT TAT TTT GAT GAG AAG GGC ATA ATG AGA ACG GGT CTT ATA Ile Lys Tyr Tyr Phe Asp Glu Lys Gly Ile Met Arg Thr Gly Leu Ile 2245 2250 2255	6768
	TCA TTT GAA AAT AAT AAT TAT TAC TTT AAT GAG AAT GGT GAA ATG CAA Ser Phe Glu Asn Asn Asn Tyr Tyr Phe Asn Glu Asn Gly Glu Met Gln 2260 2265 2270	6816
45	TTT GGT TAT ATA AAT ATA GAA GAT AAG ATG TTC TAT TTT GGT GAA GAT Phe Gly Tyr Ile Asn Ile Glu Asp Lys Met Phe Tyr Phe Gly Glu Asp 2275 2280 2285	6864
50	GGT GTC ATG CAG ATT GGA GTA TTT AAT ACA CCA GAT GGA TTT AAA TAC Gly Val Met Gln Ile Gly Val Phe Asn Thr Pro Asp Gly Phe Lys Tyr 2290 2295 2300	6912
55	TTT GCA CAT CAA AAT ACT TTG GAT GAG AAT TTT GAG GGA GAA TCA ATA Phe Ala His Gln Asn Thr Leu Asp Glu Asn Phe Glu Gly Glu Ser Ile 2305 2310 2315 2320	6960
60	AAC TAT ACT GGT TGG TTA GAT TTA GAT GAA AAG AGA TAT TAT TTT ACA Asn Tyr Thr Gly Trp Leu Asp Leu Asp Glu Lys Arg Tyr Tyr Phe Thr 2325 2330 2335	7008
	GAT GAA TAT ATT GCA GCA ACT GGT TCA GTT ATT ATT GAT GGT GAG GAG Asp Glu Tyr Ile Ala Ala Thr Gly Ser Val Ile Ile Asp Gly Glu Glu 2340 2345 2350	7056
65	TAT TAT TTT GAT CCT GAT ACA GCT CAA TTA GTG ATT AGT GAA Tyr Tyr Phe Asp Pro Asp Thr Ala Gln Leu Val Ile Ser Glu 2355 2360 2365	7098
70	TAG	7101

## (2) INFORMATION FOR SEQ ID NO:10:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2366 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

5 Met Ser Leu Val Asn Arg Lys Gln Leu Glu Lys Met Ala Asn Val Arg  
 1 5 10 15  
 15 Phe Arg Thr Gln Glu Asp Glu Tyr Val Ala Ile Leu Asp Ala Leu Glu  
 20 25 30  
 20 Glu Tyr His Asn Met Ser Glu Asn Thr Val Val Glu Lys Tyr Leu Lys  
 35 40 45  
 25 Leu Lys Asp Ile Asn Ser Leu Thr Asp Ile Tyr Ile Asp Thr Tyr Lys  
 50 55 60  
 30 Lys Ser Gly Arg Asn Lys Ala Leu Lys Lys Phe Lys Glu Tyr Leu Val  
 65 70 75 80  
 35 Thr Glu Val Leu Glu Leu Lys Asn Asn Asn Leu Thr Pro Val Glu Lys  
 85 90 95  
 40 Asn Leu His Phe Val Trp Ile Gly Gly Gln Ile Asn Asp Thr Ala Ile  
 100 105 110  
 45 Asn Tyr Ile Asn Gln Trp Lys Asp Val Asn Ser Asp Tyr Asn Val Asn  
 115 120 125  
 50 Val Phe Tyr Asp Ser Asn Ala Phe Leu Ile Asn Thr Leu Lys Lys Thr  
 130 135 140  
 55 Val Val Glu Ser Ala Ile Asn Asp Thr Leu Glu Ser Phe Arg Glu Asn  
 145 150 155 160  
 60 Leu Asn Asp Pro Arg Phe Asp Tyr Asn Lys Phe Phe Arg Lys Arg Met  
 165 170 175  
 65 Glu Ile Ile Tyr Asp Lys Gln Lys Asn Phe Ile Asn Tyr Tyr Lys Ala  
 180 185 190  
 70 Gln Arg Glu Glu Asn Pro Glu Leu Ile Ile Asp Asp Ile Val Lys Thr  
 195 200 205  
 75 Tyr Leu Ser Asn Glu Tyr Ser Lys Glu Ile Asp Glu Leu Asn Thr Tyr  
 210 215 220  
 80 Ile Glu Glu Ser Leu Asn Lys Ile Thr Gln Asn Ser Gly Asn Asp Val  
 225 230 235 240  
 85 Arg Asn Phe Glu Glu Phe Lys Asn Gly Glu Ser Phe Asn Leu Tyr Glu  
 245 250 255  
 90 Gln Glu Leu Val Glu Arg Trp Asn Leu Ala Ala Ala Ser Asp Ile Leu  
 260 265 270  
 95 Arg Ile Ser Ala Leu Lys Glu Ile Gly Gly Met Tyr Leu Asp Val Asp  
 275 280 285  
 100 Met Leu Pro Gly Ile Gln Pro Asp Leu Phe Glu Ser Ile Glu Lys Pro  
 290 295 300  
 105 Ser Ser Val Thr Val Asp Phe Trp Glu Met Thr Lys Leu Glu Ala Ile  
 305 310 315 320



Met Lys Tyr Lys Glu Tyr Ile Pro Glu Tyr Thr Ser Glu His Phe Asp  
 325 330 335  
 5 Met Leu Asp Glu Glu Val Gln Ser Ser Phe Glu Ser Val Leu Ala Ser  
 340 345 350  
 Lys Ser Asp Lys Ser Glu Ile Phe Ser Ser Leu Gly Asp Met Glu Ala  
 355 360 365  
 10 Ser Pro Leu Glu Val Lys Ile Ala Phe Asn Ser Lys Gly Ile Ile Asn  
 370 375 380  
 Gln Gly Leu Ile Ser Val Lys Asp Ser Tyr Cys Ser Asn Leu Ile Val  
 385 390 395 400  
 15 Lys Gln Ile Glu Asn Arg Tyr Lys Ile Leu Asn Asn Ser Leu Asn Pro  
 405 410 415  
 20 Ala Ile Ser Glu Asp Asn Asp Phe Asn Thr Thr Thr Asn Thr Phe Ile  
 420 425 430  
 Asp Ser Ile Met Ala Glu Ala Asn Ala Asp Asn Gly Arg Phe Met Met  
 435 440 445  
 25 Glu Leu Gly Lys Tyr Leu Arg Val Gly Phe Phe Pro Asp Val Lys Thr  
 450 455 460  
 Thr Ile Asn Leu Ser Gly Pro Glu Ala Tyr Ala Ala Tyr Gln Asp  
 465 470 475 480  
 30 Leu Leu Met Phe Lys Glu Gly Ser Met Asn Ile His Leu Ile Glu Ala  
 485 490 495  
 35 Asp Leu Arg Asn Phe Glu Ile Ser Lys Thr Asn Ile Ser Gln Ser Thr  
 500 505 510  
 Glu Gln Glu Met Ala Ser Leu Trp Ser Phe Asp Asp Ala Arg Ala Lys  
 515 520 525  
 40 Ala Gln Phe Glu Glu Tyr Lys Arg Asn Tyr Phe Glu Gly Ser Leu Gly  
 530 535 540  
 Glu Asp Asp Asn Leu Asp Phe Ser Gln Asn Ile Val Val Asp Lys Glu  
 545 550 555 560  
 45 Tyr Leu Leu Glu Lys Ile Ser Ser Leu Ala Arg Ser Ser Glu Arg Gly  
 565 570 575  
 50 Tyr Ile His Tyr Ile Val Gln Leu Gln Gly Asp Lys Ile Ser Tyr Glu  
 580 585 590  
 Ala Ala Cys Asn Leu Phe Ala Lys Thr Pro Tyr Asp Ser Val Leu Phe  
 595 600 605  
 55 Gln Lys Asn Ile Glu Asp Ser Glu Ile Ala Tyr Tyr Tyr Asn Pro Gly  
 610 615 620  
 Asp Gly Glu Ile Gln Glu Ile Asp Lys Tyr Lys Ile Pro Ser Ile Ile  
 625 630 635 640  
 60 Ser Asp Arg Pro Lys Ile Lys Leu Thr Phe Ile Gly His Gly Lys Asp  
 645 650 655  
 65 Glu Phe Asn Thr Asp Ile Phe Ala Gly Phe Asp Val Asp Ser Leu Ser  
 660 665 670  
 Thr Glu Ile Glu Ala Ala Ile Asp Leu Ala Lys Glu Asp Ile Ser Pro  
 675 680 685

Lys Ser Ile Glu Ile Asn Leu Leu Gly Cys Asn Met Phe Ser Tyr Ser  
 690 695 700  
 5 Ile Asn Val Glu Glu Thr Tyr Pro Gly Lys Leu Leu Leu Lys Val Lys  
 705 710 715 720  
 Asp Lys Ile Ser Glu Leu Met Pro Ser Ile Ser Gln Asp Ser Ile Ile  
 725 730 735  
 10 Val Ser Ala Asn Gln Tyr Glu Val Arg Ile Asn Ser Glu Gly Arg Arg  
 740 745 750  
 Glu Leu Leu Asp His Ser Gly Glu Trp Ile Asn Lys Glu Glu Ser Ile  
 755 760 765  
 15 Ile Lys Asp Ile Ser Ser Lys Glu Tyr Ile Ser Phe Asn Pro Lys Glu  
 770 775 780  
 20 Asn Lys Ile Thr Val Lys Ser Lys Asn Leu Pro Glu Leu Ser Thr Leu  
 785 790 795 800  
 Leu Gln Glu Ile Arg Asn Asn Ser Asn Ser Ser Asp Ile Glu Leu Glu  
 805 810 815  
 25 Glu Lys Val Met Leu Thr Glu Cys Glu Ile Asn Val Ile Ser Asn Ile  
 820 825 830  
 Asp Thr Gln Ile Val Glu Glu Arg Ile Glu Glu Ala Lys Asn Leu Thr  
 835 840 845  
 30 Ser Asp Ser Ile Asn Tyr Ile Lys Asp Glu Phe Lys Leu Ile Glu Ser  
 850 855 860  
 35 Ile Ser Asp Ala Leu Cys Asp Leu Lys Gln Gln Asn Glu Leu Glu Asp  
 865 870 875 880  
 Ser His Phe Ile Ser Phe Glu Asp Ile Ser Glu Thr Asp Glu Gly Phe  
 885 890 895  
 40 Ser Ile Arg Phe Ile Asn Lys Glu Thr Gly Glu Ser Ile Phe Val Glu  
 900 905 910  
 Thr Glu Lys Thr Ile Phe Ser Glu Tyr Ala Asn His Ile Thr Glu Glu  
 915 920 925  
 45 Ile Ser Lys Ile Lys Gly Thr Ile Phe Asp Thr Val Asn Gly Lys Leu  
 930 935 940  
 50 Val Lys Lys Val Asn Leu Asp Thr Thr His Glu Val Asn Thr Leu Asn  
 945 950 955 960  
 Ala Ala Phe Phe Ile Gln Ser Leu Ile Glu Tyr Asn Ser Ser Lys Glu  
 965 970 975  
 55 Ser Leu Ser Asn Leu Ser Val Ala Met Lys Val Gln Val Tyr Ala Gln  
 980 985 990  
 60 Leu Phe Ser Thr Gly Leu Asn Thr Ile Thr Asp Ala Ala Lys Val Val  
 995 1000 1005  
 Glu Leu Val Ser Thr Ala Leu Asp Glu Thr Ile Asp Leu Leu Pro Thr  
 1010 1015 1020  
 65 Leu Ser Glu Gly Leu Pro Ile Ile Ala Thr Ile Ile Asp Gly Val Ser  
 1025 1030 1035 1040  
 Leu Gly Ala Ala Ile Lys Glu Leu Ser Glu Thr Ser Asp Pro Leu Leu  
 1045 1050 1055

Arg Gln Glu Ile Glu Ala Lys Ile Gly Ile Met Ala Val Asn Leu Thr  
 1060 1065 1070  
 5 Thr Ala Thr Thr Ala Ile Ile Thr Ser Ser Leu Gly Ile Ala Ser Gly  
 1075 1080 1085  
 Phe Ser Ile Leu Leu Val Pro Leu Ala Gly Ile Ser Ala Gly Ile Pro  
 1090 1095 1100  
 10 Ser Leu Val Asn Asn Glu Leu Val Leu Arg Asp Lys Ala Thr Lys Val  
 1105 1110 1115 1120  
 Val Asp Tyr Phe Lys His Val Ser Leu Val Glu Thr Glu Gly Val Phe  
 1125 1130 1135  
 15 Thr Leu Leu Asp Asp Lys Ile Met Met Pro Gln Asp Asp Leu Val Ile  
 1140 1145 1150  
 20 Ser Glu Ile Asp Phe Asn Asn Asn Ser Ile Val Leu Gly Lys Cys Glu  
 1155 1160 1165  
 Ile Trp Arg Met Glu Gly Gly Ser Gly His Thr Val Thr Asp Asp Ile  
 1170 1175 1180  
 25 Asp His Phe Phe Ser Ala Pro Ser Ile Thr Tyr Arg Glu Pro His Leu  
 1185 1190 1195 1200  
 Ser Ile Tyr Asp Val Leu Glu Val Cln Lys Glu Glu Leu Asp Leu Ser  
 1205 1210 1215  
 30 Lys Asp Leu Met Val Leu Pro Asn Ala Pro Asn Arg Val Phe Ala Trp  
 1220 1225 1230  
 35 Glu Thr Gly Trp Thr Pro Gly Leu Arg Ser Leu Glu Asn Asp Gly Thr  
 1235 1240 1245  
 Lys Leu Leu Asp Arg Ile Arg Asp Asn Tyr Glu Gly Glu Phe Tyr Trp  
 1250 1255 1260  
 40 Arg Tyr Phe Ala Phe Ile Ala Asp Ala Leu Ile Thr Thr Leu Lys Pro  
 1265 1270 1275 1280  
 Arg Tyr Glu Asp Thr Asn Ile Arg Ile Asn Leu Asp Ser Asn Thr Arg  
 1285 1290 1295  
 45 Ser Phe Ile Val Pro Ile Ile Thr Thr Glu Tyr Ile Arg Glu Lys Leu  
 1300 1305 1310  
 50 Ser Tyr Ser Phe Tyr Gly Ser Gly Gly Thr Tyr Ala Leu Ser Leu Ser  
 1315 1320 1325  
 Gln Tyr Asn Met Gly Ile Asn Ile Glu Leu Ser Glu Ser Asp Val Trp  
 1330 1335 1340  
 55 Ile Ile Asp Val Asp Asn Val Val Arg Asp Val Thr Ile Glu Ser Asp  
 1345 1350 1355 1360  
 Lys Ile Lys Lys Gly Asp Leu Ile Glu Gly Ile Leu Ser Thr Leu Ser  
 1365 1370 1375  
 60 Ile Glu Glu Asn Lys Ile Ile Leu Asn Ser His Glu Ile Asn Phe Ser  
 1380 1385 1390  
 65 Gly Glu Val Asn Gly Ser Asn Gly Phe Val Ser Leu Thr Phe Ser Ile  
 1395 1400 1405  
 Leu Glu Gly Ile Asn Ala Ile Ile Glu Val Asp Leu Leu Ser Lys Ser  
 1410 1415 1420

Tyr Lys Leu Leu Ile Ser Gly Glu Leu Lys Ile Leu Met Leu Asn Ser  
 1425 1430 1435 1440  
 5 Asn His Ile Gln Gln Lys Ile Asp Tyr Ile Gly Phe Asn Ser Glu Leu  
 1445 1450 1455  
 Gln Lys Asn Ile Pro Tyr Ser Phe Val Asp Ser Glu Gly Lys Glu Asn  
 1460 1465 1470  
 10 Gly Phe Ile Asn Gly Ser Thr Lys Glu Gly Leu Phe Val Ser Glu Leu  
 1475 1480 1485  
 Pro Asp Val Val Leu Ile Ser Lys Val Tyr Met Asp Asp Ser Lys Pro  
 1490 1495 1500  
 15 Ser Phe Gly Tyr Tyr Ser Asn Asn Leu Lys Asp Val Lys Val Ile Thr  
 1505 1510 1515 1520  
 20 Lys Asp Asn Val Asn Ile Leu Thr Gly Tyr Tyr Leu Lys Asp Asp Ile  
 1525 1530 1535  
 Lys Ile Ser Leu Ser Leu Thr Leu Gln Asp Glu Lys Thr Ile Lys Leu  
 1540 1545 1550  
 25 Asn Ser Val His Leu Asp Glu Ser Gly Val Ala Glu Ile Leu Lys Phe  
 1555 1560 1565  
 Met Asn Arg Lys Gly Asn Thr Asn Thr Ser Asp Ser Leu Met Ser Phe  
 1570 1575 1580  
 30 Leu Glu Ser Met Asn Ile Lys Ser Ile Phe Val Asn Phe Leu Gln Ser  
 1585 1590 1595 1600  
 35 Asn Ile Lys Phe Ile Leu Asp Ala Asn Phe Ile Ile Ser Gly Thr Thr  
 1605 1610 1615  
 Ser Ile Gly Gln Phe Glu Phe Ile Cys Asp Glu Asn Asp Asn Ile Gln  
 1620 1625 1630  
 40 Pro Tyr Phe Ile Lys Phe Asn Thr Leu Glu Thr Asn Tyr Thr Leu Tyr  
 1635 1640 1645  
 Val Gly Asn Arg Gln Asn Met Ile Val Glu Pro Asn Tyr Asp Leu Asp  
 1650 1655 1660  
 45 Asp Ser Gly Asp Ile Ser Ser Thr Val Ile Asn Phe Ser Gln Lys Tyr  
 1665 1670 1675 1680  
 50 Leu Tyr Gly Ile Asp Ser Cys Val Asn Lys Val Val Ile Ser Pro Asn  
 1685 1690 1695  
 Ile Tyr Thr Asp Glu Ile Asn Ile Thr Pro Val Tyr Glu Thr Asn Asn  
 1700 1705 1710  
 55 Thr Tyr Pro Glu Val Ile Val Leu Asp Ala Asn Tyr Ile Asn Glu Lys  
 1715 1720 1725  
 Ile Asn Val Asn Ile Asn Asp Leu Ser Ile Arg Tyr Val Trp Ser Asn  
 1730 1735 1740  
 60 Asp Gly Asn Asp Phe Ile Leu Met Ser Thr Ser Glu Glu Asn Lys Val  
 1745 1750 1755 1760  
 65 Ser Gln Val Lys Ile Arg Phe Val Asn Val Phe Lys Asp Lys Thr Leu  
 1765 1770 1775  
 Ala Asn Lys Leu Ser Phe Asn Phe Ser Asp Lys Gln Asp Val Pro Val  
 1780 1785 1790

Ser Glu Ile Ile Leu Ser Phe Thr Pro Ser Tyr Tyr Glu Asp Gly Leu  
 1795 1800 1805  
 5 Ile Gly Tyr Asp Leu Gly Leu Val Ser Leu Tyr Asn Glu Lys Phe Tyr  
 1810 1815 1820  
 Ile Asn Asn Phe Gly Met Met Val Ser Gly Leu Ile Tyr Ile Asn Asp  
 1825 1830 1835 1840  
 10 Ser Leu Tyr Tyr Phe Lys Pro Pro Val Asn Asn Leu Ile Thr Gly Phe  
 1845 1850 1855  
 Val Thr Val Gly Asp Asp Lys Tyr Tyr Phe Asn Pro Ile Asn Gly Gly  
 1860 1865 1870  
 15 Ala Ala Ser Ile Gly Glu Thr Ile Ile Asp Asp Lys Asn Tyr Tyr Phe  
 1875 1880 1885  
 Asn Gln Ser Gly Val Leu Gln Thr Gly Val Phe Ser Thr Glu Asp Gly  
 1890 1895 1900  
 Phe Lys Tyr Phe Ala Pro Ala Asn Thr Leu Asp Glu Asn Leu Glu Gly  
 1905 1910 1915 1920  
 25 Glu Ala Ile Asp Phe Thr Gly Lys Leu Ile Ile Asp Glu Asn Ile Tyr  
 1925 1930 1935  
 Tyr Phe Asp Asp Asn Tyr Arg Gly Ala Val Glu Trp Lys Glu Leu Asp  
 1940 1945 1950  
 30 Gly Glu Met His Tyr Phe Ser Pro Glu Thr Gly Lys Ala Phe Lys Gly  
 1955 1960 1965  
 Leu Asn Gln Ile Gly Asp Tyr Lys Tyr Tyr Phe Asn Ser Asp Gly Val  
 1970 1975 1980  
 Met Gln Lys Gly Phe Val Ser Ile Asn Asp Asn Lys His Tyr Phe Asp  
 1985 1990 1995 2000  
 40 Asp Ser Gly Val Met Lys Val Gly Tyr Thr Glu Ile Asp Gly Lys His  
 2005 2010 2015  
 Phe Tyr Phe Ala Glu Asn Gly Glu Met Gln Ile Gly Val Phe Asn Thr  
 2020 2025 2030  
 45 Glu Asp Gly Phe Lys Tyr Phe Ala His His Asn Glu Asp Leu Gly Asn  
 2035 2040 2045  
 Glu Glu Gly Glu Glu Ile Ser Tyr Ser Gly Ile Leu Asn Phe Asn Asn  
 2050 2055 2060  
 Lys Ile Tyr Tyr Phe Asp Asp Ser Phe Thr Ala Val Val Gly Trp Lys  
 2065 2070 2075 2080  
 55 Asp Leu Glu Asp Gly Ser Lys Tyr Tyr Phe Asp Glu Asp Thr Ala Glu  
 2085 2090 2095  
 Ala Tyr Ile Gly Leu Ser Leu Ile Asn Asp Gly Gln Tyr Tyr Phe Asn  
 2100 2105 2110  
 60 Asp Asp Gly Ile Met Gln Val Gly Phe Val Thr Ile Asn Asp Lys Val  
 2115 2120 2125  
 Phe Tyr Phe Ser Asp Ser Gly Ile Ile Glu Ser Gly Val Gln Asn Ile  
 2130 2135 2140  
 Asp Asp Asn Tyr Phe Tyr Ile Asp Asp Asn Gly Ile Val Gln Ile Gly  
 2145 2150 2155 2160  
 70 Val Phe Asp Thr Ser Asp Gly Tyr Lys Tyr Phe Ala Pro Ala Asn Thr

[illegible]

40 (2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 19 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

50) (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TAGAAAAAAT GGCAAATGT

(2) INFORMATION FOR SEQ ID NO:12:

55 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 21 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

65 TTTCATCTTG TAGAGTCAAA G

(2) INFORMATION FOR SEQ ID NO:13:

70) (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 22 base pairs

(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: DNA (genomic)  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:  
GATGCCACAA GATGATTAG TG 22

10 (2) INFORMATION FOR SEQ ID NO:14:  
(i) SEQUENCE CHARACTERISTICS:  
15 (A) LENGTH: 22 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ii) MOLECULE TYPE: DNA (genomic)  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:  
CTAATTGAGC TGTATCAGGA TC 22

25 (2) INFORMATION FOR SEQ ID NO:15:  
(i) SEQUENCE CHARACTERISTICS:  
30 (A) LENGTH: 27 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ii) MOLECULE TYPE: DNA (genomic)  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:  
CGGAATTCCT AGAAAAAATG GCAAATG 27

40 (2) INFORMATION FOR SEQ ID NO:16:  
(i) SEQUENCE CHARACTERISTICS:  
45 (A) LENGTH: 26 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ii) MOLECULE TYPE: DNA (genomic)  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:  
50 GCTCTAGAAT GACCATAAGC TAGCCA 26

(2) INFORMATION FOR SEQ ID NO:17:  
55 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 27 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
60 (ii) MOLECULE TYPE: DNA (genomic)  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:  
65 CGGAATTCGA GTTGGTAGAA AGGTGGA 27

(2) INFORMATION FOR SEQ ID NO:18:  
70 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 27 base pairs

(B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

10 CGGAATTCGG TTATTATCTT AAGGATG

27

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

15 (A) LENGTH: 28 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CGGAATTCTT GATAACTGGA TTTGTGAC

28

25 (2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

30 (A) LENGTH: 511 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: unknown  
 (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Leu Ile Thr Gly Phe Val Thr Val Gly Asp Asp Lys Tyr Tyr Phe Asn  
 1 5 10 15

40 Pro Ile Asn Gly Gly Ala Ala Ser Ile Gly Glu Thr Ile Ile Asp Asp  
 20 25 30

45 Lys Asn Tyr Tyr Phe Asn Gln Ser Gly Val Leu Gln Thr Gly Val Phe  
 35 40 45

Ser Thr Glu Asp Gly Phe Lys Tyr Phe Ala Pro Ala Asn Thr Leu Asp  
 50 55 60

50 Glu Asn Leu Glu Gly Glu Ala Ile Asp Phe Thr Gly Lys Leu Ile Ile  
 65 70 75 80

Asp Glu Asn Ile Tyr Tyr Phe Asp Asp Asn Tyr Arg Gly Ala Val Glu  
 85 90 95

55 Trp Lys Glu Leu Asp Gly Glu Met His Tyr Phe Ser Pro Glu Thr Gly  
 100 105 110

60 Lys Ala Phe Lys Gly Leu Asn Gln Ile Gly Asp Tyr Lys Tyr Tyr Phe  
 115 120 125

Asn Ser Asp Gly Val Met Gln Lys Gly Phe Val Ser Ile Asn Asp Asn  
 130 135 140

65 Lys His Tyr Phe Asp Asp Ser Gly Val Met Lys Val Gly Tyr Thr Glu  
 145 150 155 160

Ile Asp Gly Lys His Phe Tyr Phe Ala Glu Asn Gly Glu Met Gln Ile  
 165 170 175

70 Gly Val Phe Asn Thr Glu Asp Gly Phe Lys Tyr Phe Ala His His Asn



	180	185	190
5	Glu Asp Leu Gly Asn Glu Glu Gly Glu Glu Ile Ser Tyr Ser Gly Ile 195 200 205		
	Leu Asn Phe Asn Asn Lys Ile Tyr Tyr Phe Asp Asp Ser Phe Thr Ala 210 215 220		
10	Val Val Gly Trp Lys Asp Leu Glu Asp Gly Ser Lys Tyr Tyr Phe Asp 225 230 235 240		
	Glu Asp Thr Ala Glu Ala Tyr Ile Gly Leu Ser Leu Ile Asn Asp Gly 245 250 255		
15	Gln Tyr Tyr Phe Asn Asp Asp Gly Ile Met Gln Val Gly Phe Val Thr 260 265 270		
	Ile Asn Asp Lys Val Phe Tyr Phe Ser Asp Ser Gly Ile Ile Glu Ser 275 280 285		
20	Gly Val Gln Asn Ile Asp Asp Asn Tyr Phe Tyr Ile Asp Asp Asn Gly 290 295 300		
25	Ile Val Gln Ile Gly Val Phe Asp Thr Ser Asp Gly Tyr Lys Tyr Phe 305 310 315 320		
	Ala Pro Ala Asn Thr Val Asn Asp Asn Ile Tyr Gly Gln Ala Val Glu 325 330 335		
30	Tyr Ser Gly Leu Val Arg Val Gly Glu Asp Val Tyr Tyr Phe Gly Glu 340 345 350		
	Thr Tyr Thr Ile Glu Thr Gly Trp Ile Tyr Asp Met Glu Asn Glu Ser 355 360 365		
35	Asp Lys Tyr Tyr Phe Asn Pro Glu Thr Lys Lys Ala Cys Lys Gly Ile 370 375 380		
40	Asn Leu Ile Asp Asp Ile Lys Tyr Tyr Phe Asp Glu Lys Gly Ile Met 385 390 395 400		
	Arg Thr Gly Leu Ile Ser Phe Glu Asn Asn Tyr Tyr Phe Asn Glu 405 410 415		
45	Asn Gly Glu Met Gln Phe Gly Tyr Ile Asn Ile Glu Asp Lys Met Phe 420 425 430		
	Tyr Phe Gly Glu Asp Gly Val Met Gln Ile Gly Val Phe Asn Thr Pro 435 440 445		
50	Asp Gly Phe Lys Tyr Phe Ala His Gln Asn Thr Leu Asp Glu Asn Phe 450 455 460		
55	Glu Gly Glu Ser Ile Asn Tyr Thr Gly Trp Leu Asp Leu Asp Glu Lys 465 470 475 480		
	Arg Tyr Tyr Phe Thr Asp Glu Tyr Ile Ala Ala Thr Gly Ser Val Ile 485 490 495		
60	Ile Asp Gly Glu Glu Tyr Tyr Phe Asp Pro Asp Thr Ala Gln Leu 500 505 510		

## (2) INFORMATION FOR SEQ ID NO:21:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 608 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: unknown  
 (D) TOPOLOGY: unknown

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Ser Glu Glu Asn Lys Val Ser Gln Val Lys Ile Arg Phe Val Asn Val  
 1 5 10 15  
 Phe Lys Asp Lys Thr Leu Ala Asn Lys Leu Ser Phe Asn Phe Ser Asp  
 20 25 30  
 Lys Gln Asp Val Pro Val Ser Glu Ile Ile Leu Ser Phe Thr Pro Ser  
 35 40 45  
 Tyr Tyr Glu Asp Gly Leu Ile Gly Tyr Asp Leu Gly Leu Val Ser Leu  
 50 55 60  
 Tyr Asn Glu Lys Phe Tyr Ile Asn Asn Phe Gly Met Met Val Ser Gly  
 65 70 75 80  
 Leu Ile Tyr Ile Asn Asp Ser Leu Tyr Tyr Phe Lys Pro Pro Val Asn  
 85 90 95  
 Asn Leu Ile Thr Gly Phe Val Thr Val Gly Asp Asp Lys Tyr Tyr Phe  
 100 105 110  
 Asn Pro Ile Asn Gly Gly Ala Ala Ser Ile Gly Glu Thr Ile Ile Asp  
 115 120 125  
 Asp Lys Asn Tyr Tyr Phe Asn Gln Ser Gly Val Leu Gln Thr Gly Val  
 130 135 140  
 Phe Ser Thr Glu Asp Gly Phe Lys Tyr Phe Ala Pro Ala Asn Thr Leu  
 145 150 155 160  
 Asp Glu Asn Leu Glu Gly Glu Ala Ile Asp Phe Thr Gly Lys Leu Ile  
 165 170 175  
 Ile Asp Glu Asn Ile Tyr Tyr Phe Asp Asp Asn Tyr Arg Gly Ala Val  
 180 185 190  
 Glu Trp Lys Glu Leu Asp Gly Glu Met His Tyr Phe Ser Pro Glu Thr  
 195 200 205  
 Gly Lys Ala Phe Lys Gly Leu Asn Gln Ile Gly Asp Tyr Lys Tyr Tyr  
 210 215 220  
 Phe Asn Ser Asp Gly Val Met Gln Lys Gly Phe Val Ser Ile Asn Asp  
 225 230 235 240  
 Asn Lys His Tyr Phe Asp Asp Ser Gly Val Met Lys Val Gly Tyr Thr  
 245 250 255  
 Glu Ile Asp Gly Lys His Phe Tyr Phe Ala Glu Asn Gly Glu Met Gln  
 260 265 270  
 Ile Gly Val Phe Asn Thr Glu Asp Gly Phe Lys Tyr Phe Ala His His  
 275 280 285  
 Asn Glu Asp Leu Gly Asn Glu Glu Gly Glu Glu Ile Ser Tyr Ser Gly  
 290 295 300  
 Ile Leu Asn Phe Asn Asn Lys Ile Tyr Tyr Phe Asp Asp Ser Phe Thr

	305	310	315	320
5	Ala Val Val Gly Trp 325	Lys Asp Leu Glu Asp Gly Ser Lys Tyr Tyr Phe 335		
10	Asp Glu Asp Thr Ala Glu Ala Tyr Ile Gly Leu Ser Leu Ile Asn Asp 340	345	350	
15	Gly Gln Tyr Tyr Phe Asn Asp Asp Gly Ile Met Gln Val Gly Phe Val 355	360	365	
20	Thr Ile Asn Asp Lys Val Phe Tyr Phe Ser Asp Ser Gly Ile Ile Glu 370	375	380	
25	Ser Gly Val Gln Asn Ile Asp Asp Asn Tyr Phe Tyr Ile Asp Asp Asn 385	390	395	400
30	Gly Ile Val Gln Ile Gly Val Phe Asp Thr Ser Asp Gly Tyr Lys Tyr 405	410	415	
35	Phe Ala Pro Ala Asn Thr Val Asn Asp Asn Ile Tyr Gly Gln Ala Val 420	425	430	
40	Glu Tyr Ser Gly Leu Val Arg Val Gly Glu Asp Val Tyr Tyr Phe Gly 435	440	445	
45	Glu Thr Tyr Thr Ile Glu Thr Gly Trp Ile Tyr Asp Met Glu Asn Glu 450	455	460	
50	Ser Asp Lys Tyr Tyr Phe Asn Pro Glu Thr Lys Lys Ala Cys Lys Gly 465	470	475	480
55	Ile Asn Leu Ile Asp Asp Ile Lys Tyr Tyr Phe Asp Glu Lys Gly Ile 485	490	495	
60	Met Arg Thr Gly Leu Ile Ser Phe Glu Asn Asn Asn Tyr Tyr Phe Asn 500	505	510	
65	Glu Asn Gly Glu Met Gln Phe Gly Tyr Ile Asn Ile Glu Asp Lys Met 515	520	525	
70	Phe Tyr Phe Gly Glu Asp Gly Val Met Gln Ile Gly Val Phe Asn Thr 530	535	540	
75	Pro Asp Gly Phe Lys Tyr Phe Ala His Gln Asn Thr Leu Asp Glu Asn 545	550	555	560
80	Phe Glu Gly Glu Ser Ile Asn Tyr Thr Gly Trp Leu Asp Leu Asp Glu 565	570	575	
85	Lys Arg Tyr Tyr Phe Thr Asp Glu Tyr Ile Ala Ala Thr Gly Ser Val 580	585	590	
90	Ile Ile Asp Gly Glu Glu Tyr Tyr Phe Asp Pro Asp Thr Ala Gln Leu 595	600	605	

(2) INFORMATION FOR SEQ ID NO:22:

60 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 1330 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear

65 (ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:  
 (A) NAME/KEY: CDS  
 (B) LOCATION: 1..1314

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

5	ATG GCT CGT CTG CTG TCT ACC TTC ACT GAA TAC ATC AAG AAC ATC ATC Met Ala Arg Leu Leu Ser Thr Phe Thr Glu Tyr Ile Lys Asn Ile Ile	48
10	AAT ACC TCC ATC CTG AAC CTG CGC TAC GAA TCC AAT CAC CTG ATC GAC Asn Thr Ser Ile Leu Asn Leu Arg Tyr Glu Ser Asn His Leu Ile Asp	96
15	CTG TCT CGC TAC GCT TCC AAA ATC AAC ATC GGT TCT AAA GTT AAC TTC Leu Ser Arg Tyr Ala Ser Lys Ile Asn Ile Gly Ser Lys Val Asn Phe	144
20	GAT CCG ATC GAC AAG AAT CAG ATC CAG CTG TTC AAT CTG GAA TCT TCC Asp Pro Ile Asp Lys Asn Gln Ile Gln Leu Phe Asn Leu Glu Ser Ser	192
25	AAA ATC GAA GTT ATC CTG AAG AAT GCT ATC GTA TAC AAC TCT ATG TAC Lys Ile Glu Val Ile Leu Lys Asn Ala Ile Val Tyr Asn Ser Met Tyr	240
30	GAA AAC TTC TCC ACC TCC TTC TGG ATC CGT ATC CCG AAA TAC TTC AAC Glu Asn Phe Ser Thr Ser Phe Trp Ile Arg Ile Pro Lys Tyr Phe Asn	288
35	TCC ATC TCT CTG AAC AAT GAA TAC ACC ATC ATC AAC TGC ATG GAA AAC Ser Ile Ser Leu Asn Asn Glu Tyr Thr Ile Ile Asn Cys Met Glu Asn	336
40	AAT TCT GGT TGG AAA GTA TCT CTG AAC TAC GGT GAA ATC ATC TGG ACT Asn Ser Gly Trp Lys Val Ser Leu Asn Tyr Gly Glu Ile Ile Trp Thr	384
45	CTG CAG GAC ACT CAG GAA ATC AAA CAG CGT GTT GTA TTC AAA TAC TCT Leu Gln Asp Thr Gln Glu Ile Lys Gln Arg Val Phe Lys Tyr Ser	432
50	CAG ATG ATC AAC ATC TCT GAC TAC ATC AAT CGC TGG ATC TTC GTT ACC Gln Met Ile Asn Ile Ser Asp Tyr Ile Asn Arg Trp Ile Phe Val Thr	480
55	ATC ACC AAC AAT CGT CTG AAT AAC TCC AAA ATC TAC ATC AAC GGC CGT Ile Thr Asn Asn Arg Leu Asn Asn Ser Lys Ile Tyr Ile Asn Gly Arg	528
60	CTG ATC GAC CAG AAA CCG ATC TCC AAT CTG GGT AAC ATC CAC GCT TCT Leu Ile Asp Gln Lys Pro Ile Ser Asn Leu Gly Asn Ile His Ala Ser	576
65	AAT AAC ATC ATG TTC AAA CTG GAC GGT TGT CGT GAC ACT CAC CGC TAC Asn Asn Ile Met Phe Lys Leu Asp Gly Cys Arg Asp Thr His Arg Tyr	624
70	ATC TGG ATC AAA TAC TTC AAT CTG TTC GAC AAA GAA CTG AAC GAA AAA Ile Trp Ile Lys Tyr Phe Asn Leu Phe Asp Lys Glu Leu Asn Glu Lys	672
	GAA ATC AAA GAC CTG TAC GAC AAC CAG TCC AAT TCT GGT ATC CTG AAA Glu Ile Lys Asp Leu Tyr Asp Asn Gln Ser Asn Ser Gly Ile Leu Lys	720
	GAC TTC TGG GGT GAC TAC CTG CAG TAC GAC AAA CCG TAC TAC ATG CTG Asp Phe Trp Gly Asp Tyr Leu Gln Tyr Asp Lys Pro Tyr Tyr Met Leu	768
	AAT CTG TAC GAT CCG AAC AAA TAC GTT GAC GTC AAC AAT GTA GGT ATC Asn Leu Tyr Asp Pro Asn Lys Tyr Val Asp Val Asn Asn Val Gly Ile	816

	CGC GGT TAC ATG TAC CTG AAA GGT CCG CGT GGT TCT GTT ATG ACT ACC	864
	Arg Gly Tyr Met Tyr Leu Lys Gly Pro Arg Gly Ser Val Met Thr Thr	
	275 280 285	
5	AAC ATC TAC CTG AAC TCT TCC CTG TAC CGT GGT ACC AAA TTC ATC ATC	912
	Asn Ile Tyr Leu Asn Ser Ser Leu Tyr Arg Gly Thr Lys Phe Ile Ile	
	290 295 300	
10	AAG AAA TAC GCG TCT GGT AAC AAG GAC AAT ATC GTT CGC AAC AAT GAT	960
	Lys Lys Tyr Ala Ser Gly Asn Lys Asp Asn Ile Val Arg Asn Asn Asp	
	305 310 315 320	
15	CGT GTA TAC ATC AAT GTT GTA GTT AAG AAC AAA GAA TAC CGT CTG GCT	1008
	Arg Val Tyr Ile Asn Val Val Val Lys Asn Lys Glu Tyr Arg Leu Ala	
	325 330 335	
20	ACC AAT GCT TCT CAG GCT GGT GTA GAA AAG ATC TTG TCT GCT CTG GAA	1056
	Thr Asn Ala Ser Gln Ala Gly Val Glu Lys Ile Leu Ser Ala Leu Glu	
	340 345 350	
25	ATC CCG GAC GTT GGT AAT CTG TCT CAG GTA GTT GTA ATG AAA TCC AAG	1104
	Ile Pro Asp Val Gly Asn Leu Ser Gln Val Val Val Met Lys Ser Lys	
	355 360 365	
30	AAC GAC CAG GGT ATC ACT AAC AAA TGC AAA ATG AAT CTG CAG GAC AAC	1152
	Asn Asp Gln Gly Ile Thr Asn Lys Cys Lys Met Asn Leu Gln Asp Asn	
	370 375 380	
35	AAT GGT AAC GAT ATC GGT TTC ATC GGT TTC CAC CAG TTC AAC AAT ATC	1200
	Asn Gly Asn Asp Ile Gly Phe Ile Gly Phe His Gln Phe Asn Asn Ile	
	385 390 395 400	
40	GCT AAA CTG GTT GCT TCC AAC TGG TAC AAT CGT CAG ATC GAA CGT TCC	1248
	Ala Lys Leu Val Ala Ser Asn Trp Tyr Asn Arg Gln Ile Glu Arg Ser	
	405 410 415	
45	TCT CGC ACT CTG GGT TGC TCT TGG GAG TTC ATC CCG GTT GAT GAC GGT	1296
	Ser Arg Thr Leu Gly Cys Ser Trp Glu Phe Ile Pro Val Asp Asp Gly	
	420 425 430	
50	TGG GGT GAA CGT CCG CTG TAACCCGGA AAGCTT	1330
	Trp Gly Glu Arg Pro Leu	
	435	
55	(2) INFORMATION FOR SEQ ID NO:23:	
	(i) SEQUENCE CHARACTERISTICS:	
	(A) LENGTH: 438 amino acids	
	(B) TYPE: amino acid	
	(D) TOPOLOGY: linear	
60	(ii) MOLECULE TYPE: protein	
65	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:	
	Met Ala Arg Leu Leu Ser Thr Phe Thr Glu Tyr Ile Lys Asn Ile Ile	
	1 5 10 15	
70	Asn Thr Ser Ile Leu Asn Leu Arg Tyr Glu Ser Asn His Leu Ile Asp	
	20 25 30	
	Leu Ser Arg Tyr Ala Ser Lys Ile Asn Ile Gly Ser Lys Val Asn Phe	
	35 40 45	
75	Asp Pro Ile Asp Lys Asn Gln Ile Gln Leu Phe Asn Leu Glu Ser Ser	
	50 55 60	
80	Lys Ile Glu Val Ile Leu Lys Asn Ala Ile Val Tyr Asn Ser Met Tyr	
	65 70 75 80	

	Glu	Asn	Phe	Ser	Thr	Ser	Phe	Trp	Ile	Arg	Ile	Pro	Lys	Tyr	Phe	Asn
					85					90					95	
5	Ser	Ile	Ser	Leu	Asn	Asn	Glu	Tyr	Thr	Ile	Ile	Asn	Cys	Met	Glu	Asn
				100					105					110		
	Asn	Ser	Gly	Trp	Lys	Val	Ser	Leu	Asn	Tyr	Gly	Glu	Ile	Ile	Trp	Thr
			115					120					125			
10	Leu	Gln	Asp	Thr	Gln	Glu	Ile	Lys	Gln	Arg	Val	Val	Phe	Lys	Tyr	Ser
		130					135					140				
	Gln	Met	Ile	Asn	Ile	Ser	Asp	Tyr	Ile	Asn	Arg	Trp	Ile	Phe	Val	Thr
	145					150					155					160
15	Ile	Thr	Asn	Asn	Arg	Leu	Asn	Asn	Ser	Lys	Ile	Tyr	Ile	Asn	Gly	Arg
					165					170					175	
20	Leu	Ile	Asp	Gln	Lys	Pro	Ile	Ser	Asn	Leu	Gly	Asn	Ile	His	Ala	Ser
				180					185					190		
	Asn	Asn	Ile	Met	Phe	Lys	Leu	Asp	Gly	Cys	Arg	Asp	Thr	His	Arg	Tyr
			195					200					205			
25	Ile	Trp	Ile	Lys	Tyr	Phe	Asn	Leu	Phe	Asp	Lys	Glu	Leu	Asn	Glu	Lys
		210					215					220				
	Glu	Ile	Lys	Asp	Leu	Tyr	Asp	Asn	Gln	Ser	Asn	Ser	Gly	Ile	Leu	Lys
	225					230					235					240
30	Asp	Phe	Trp	Gly	Asp	Tyr	Leu	Gln	Tyr	Asp	Lys	Pro	Tyr	Tyr	Met	Leu
					245					250					255	
35	Asn	Leu	Tyr	Asp	Pro	Asn	Lys	Tyr	Val	Asp	Val	Asn	Asn	Val	Gly	Ile
				260					265					270		
	Arg	Gly	Tyr	Met	Tyr	Leu	Lys	Gly	Pro	Arg	Gly	Ser	Val	Met	Thr	Thr
			275					280					285			
40	Asn	Ile	Tyr	Leu	Asn	Ser	Ser	Leu	Tyr	Arg	Gly	Thr	Lys	Phe	Ile	Ile
		290					295					300				
	Lys	Lys	Tyr	Ala	Ser	Gly	Asn	Lys	Asp	Asn	Ile	Val	Arg	Asn	Asn	Asp
	305					310					315					320
45	Arg	Val	Tyr	Ile	Asn	Val	Val	Val	Lys	Asn	Lys	Glu	Tyr	Arg	Leu	Ala
					325					330					335	
50	Thr	Asn	Ala	Ser	Gln	Ala	Gly	Val	Glu	Lys	Ile	Leu	Ser	Ala	Leu	Glu
				340					345					350		
	Ile	Pro	Asp	Val	Gly	Asn	Leu	Ser	Gln	Val	Val	Val	Met	Lys	Ser	Lys
			355					360					365			
55	Asn	Asp	Gln	Gly	Ile	Thr	Asn	Lys	Cys	Lys	Met	Asn	Leu	Gln	Asp	Asn
		370					375					380				
	Asn	Gly	Asn	Asp	Ile	Gly	Phe	Ile	Gly	Phe	His	Gln	Phe	Asn	Asn	Ile
					390						395					400
60	Ala	Lys	Leu	Val	Ala	Ser	Asn	Trp	Tyr	Asn	Arg	Gln	Ile	Glu	Arg	Ser
					405											

70 (2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 23 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: unknown  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

Met Gly His His His His His His His His His Ser Ser Gly His  
 1 5 10 15  
 Ile Glu Gly Arg His Met Ala  
 20

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 1402 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:  
 (A) NAME/KEY: CDS  
 (B) LOCATION: 1..1386

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

ATG GGC CAT CAT CAT CAT CAT CAT CAT CAT CAC AGC AGC GGC CAT 48  
 Met Gly His His His His His His His His His Ser Ser Gly His  
 1 5 10 15  
 ATC GAA GGT CGT CAT ATG GCT AGC ATG GCT CGT CTG CTG TCT ACC TTC 96  
 Ile Glu Gly Arg His Met Ala Ser Met Ala Arg Leu Leu Ser Thr Phe  
 20 25 30  
 ACT GAA TAC ATC AAG AAC ATC ATC AAT ACC TCC ATC CTG AAC CTG CGC 144  
 Thr Glu Tyr Ile Lys Asn Ile Ile Asn Thr Ser Ile Leu Asn Leu Arg  
 35 40 45  
 TAC GAA TCC AAT CAC CTG ATC GAC CTG TCT CGC TAC GCT TCC AAA ATC 192  
 Tyr Glu Ser Asn His Leu Ile Asp Leu Ser Arg Tyr Ala Ser Lys Ile  
 50 55 60  
 AAC ATC GGT TCT AAA GTT AAC TTC GAT CCG ATC GAC AAG AAT CAG ATC 240  
 Asn Ile Gly Ser Lys Val Asn Phe Asp Pro Ile Asp Lys Asn Gln Ile  
 65 70 75 80  
 CAG CTG TTC AAT CTG GAA TCT TCC AAA ATC GAA GTT ATC CTG AAG AAT 288  
 Gln Leu Phe Asn Leu Glu Ser Ser Lys Ile Glu Val Ile Leu Lys Asn  
 85 90 95  
 GCT ATC GTA TAC AAC TCT ATG TAC GAA AAC TTC TCC ACC TCC TTC TGG 336  
 Ala Ile Val Tyr Asn Ser Met Tyr Glu Asn Phe Ser Thr Ser Phe Trp  
 100 105 110  
 ATC CGT ATC CCG AAA TAC TTC AAC TCC ATC TCT CTG AAC AAT GAA TAC 384  
 Ile Arg Ile Pro Lys Tyr Phe Asn Ser Ile Ser Leu Asn Asn Glu Tyr  
 115 120 125  
 ACC ATC ATC AAC TGC ATG GAA AAC AAT TCT GGT TGG AAA GTA TCT CTG 432  
 Thr Ile Ile Asn Cys Met Glu Asn Asn Ser Gly Trp Lys Val Ser Leu  
 130 135 140  
 AAC TAC GGT GAA ATC ATC TGG ACT CTG CAG GAC ACT CAG GAA ATC AAA 480  
 Asn Tyr Gly Glu Ile Ile Trp Thr Leu Gln Asp Thr Gln Glu Ile Lys

	145				150				155				160		
5	CAG CGT GTT GTA TTC AAA TAC TCT CAG ATG ATC AAC ATC TCT GAC TAC														528
	Gln Arg Val Val Phe Lys Tyr Ser Gln Met Ile Asn Ile Ser Asp Tyr														
				165				170					175		
10	ATC AAT CGC TGG ATC TTC GTT ACC ATC ACC AAC AAT CGT CTG AAT AAC														576
	Ile Asn Arg Trp Ile Phe Val Thr Ile Thr Asn Asn Arg Leu Asn Asn														
			180					185					190		
	TCC AAA ATC TAC ATC AAC GGC CGT CTG ATC GAC CAG AAA CCG ATC TCC														624
	Ser Lys Ile Tyr Ile Asn Gly Arg Leu Ile Asp Gln Lys Pro Ile Ser														
			195				200					205			
15	AAT CTG GGT AAC ATC CAC GCT TCT AAT AAC ATC ATG TTC AAA CTG GAC														672
	Asn Leu Gly Asn Ile His Ala Ser Asn Asn Ile Met Phe Lys Leu Asp														
			210			215					220				
20	GGT TGT CGT GAC ACT CAC CGC TAC ATC TGG ATC AAA TAC TTC AAT CTG														720
	Gly Cys Arg Asp Thr His Arg Tyr Ile Trp Ile Lys Tyr Phe Asn Leu														
					230				235					240	
25	TTC GAC AAA GAA CTG AAC GAA AAA GAA ATC AAA GAC CTG TAC GAC AAC														768
	Phe Asp Lys Glu Leu Asn Glu Lys Glu Ile Lys Asp Leu Tyr Asp Asn														
				245					250					255	
30	CAG TCC AAT TCT GGT ATC CTG AAA GAC TTC TGG GGT GAC TAC CTG CAG														816
	Gln Ser Asn Ser Gly Ile Leu Lys Asp Phe Trp Gly Asp Tyr Leu Gln														
			260					265					270		
	TAC GAC AAA CCG TAC TAC ATG CTG AAT CTG TAC GAT CCG AAC AAA TAC														864
	Tyr Asp Lys Pro Tyr Tyr Met Leu Asn Leu Tyr Asp Pro Asn Lys Tyr														
			275				280					285			
35	GTT GAC GTC AAC AAT GTA GGT ATC CGC GGT TAC ATG TAC CTG AAA GGT														912
	Val Asp Val Asn Asn Val Gly Ile Arg Gly Tyr Met Tyr Leu Lys Gly														
			290			295					300				
40	CCG CGT GGT TCT GTT ATG ACT ACC AAC ATC TAC CTG AAC TCT TCC CTG														960
	Pro Arg Gly Ser Val Met Thr Thr Asn Ile Tyr Leu Asn Ser Ser Leu														
					310				315					320	
45	TAC CGT GGT ACC AAA TTC ATC ATC AAG AAA TAC GCG TCT GGT AAC AAG														1008
	Tyr Arg Gly Thr Lys Phe Ile Ile Lys Lys Tyr Ala Ser Gly Asn Lys														
				325					330					335	
50	GAC AAT ATC GTT CGC AAC AAT GAT CGT GTA TAC ATC AAT GTT GTA GTT														1056
	Asp Asn Ile Val Arg Asn Asn Asp Arg Val Tyr Ile Asn Val Val Val														
				340				345					350		
	AAG AAC AAA GAA TAC CGT CTG GCT ACC AAT GCT TCT CAG GCT GGT GTA														1104
	Lys Asn Lys Glu Tyr Arg Leu Ala Thr Asn Ala Ser Gln Ala Gly Val														
			355				360					365			
55	GAA AAG ATC TTG TCT GCT CTG GAA ATC CCG GAC GTT GGT AAT CTG TCT														1152
	Glu Lys Ile Leu Ser Ala Leu Glu Ile Pro Asp Val Gly Asn Leu Ser														
			370			375					380				
60	CAG GTA GTT GTA ATG AAA TCC AAG AAC GAC CAG GGT ATC ACT AAC AAA														1200
	Gln Val Val Val Met Lys Ser Lys Asn Asp Gln Gly Ile Thr Asn Lys														
					390				395					400	
65	TGC AAA ATG AAT CTG CAG GAC AAC AAT GGT AAC GAT ATC GGT TTC ATC														1248
	Cys Lys Met Asn Leu Gln Asp Asn Asn Gly Asn Asp Ile Gly Phe Ile														
				405				410					415		



	GGT TTC CAC CAG TTC AAC AAT ATC GCT AAA CTG GTT GCT TCC AAC TGG	1296
	Gly Phe His Gln Phe Asn Asn Ile Ala Lys Leu Val Ala Ser Asn Trp	
	420 425 430	
5	TAC AAT CGT CAG ATC GAA CGT TCC TCT CGC ACT CTG GGT TGC TCT TGG	1344
	Tyr Asn Arg Gln Ile Glu Arg Ser Ser Arg Thr Leu Gly Cys Ser Trp	
	435 440 445	
10	GAG TTC ATC CCG GTT GAT GAC GGT TGG GGT GAA CGT CCG CTG	1386
	Glu Phe Ile Pro Val Asp Asp Gly Trp Gly Glu Arg Pro Leu	
	450 455 460	
	TAACCCGGGA AAGCTT	1402
15	(2) INFORMATION FOR SEQ ID NO:26:	
	(i) SEQUENCE CHARACTERISTICS:	
	(A) LENGTH: 462 amino acids	
20	(B) TYPE: amino acid	
	(D) TOPOLOGY: linear	
	(ii) MOLECULE TYPE: protein	
25	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:	
	Met Gly His His His His His His His His His His Ser Ser Gly His	
	1 5 10 15	
30	Ile Glu Gly Arg His Met Ala Ser Met Ala Arg Leu Leu Ser Thr Phe	
	20 25 30	
	Thr Glu Tyr Ile Lys Asn Ile Ile Asn Thr Ser Ile Leu Asn Leu Arg	
	35 40 45	
35	Tyr Glu Ser Asn His Leu Ile Asp Leu Ser Arg Tyr Ala Ser Lys Ile	
	50 55 60	
	Asn Ile Gly Ser Lys Val Asn Phe Asp Pro Ile Asp Lys Asn Gln Ile	
40	65 70 75 80	
	Gln Leu Phe Asn Leu Glu Ser Ser Lys Ile Glu Val Ile Leu Lys Asn	
	85 90 95	
45	Ala Ile Val Tyr Asn Ser Met Tyr Glu Asn Phe Ser Thr Ser Phe Trp	
	100 105 110	
	Ile Arg Ile Pro Lys Tyr Phe Asn Ser Ile Ser Leu Asn Asn Glu Tyr	
	115 120 125	
50	Thr Ile Ile Asn Cys Met Glu Asn Asn Ser Gly Trp Lys Val Ser Leu	
	130 135 140	
	Asn Tyr Gly Glu Ile Ile Trp Thr Leu Gln Asp Thr Gln Glu Ile Lys	
55	145 150 155 160	
	Gln Arg Val Val Phe Lys Tyr Ser Gln Met Ile Asn Ile Ser Asp Tyr	
	165 170 175	
60	Ile Asn Arg Trp Ile Phe Val Thr Ile Thr Asn Asn Arg Leu Asn Asn	
	180 185 190	

Ser Lys Ile Tyr Ile Asn Gly Arg Leu Ile Asp Gln Lys Pro Ile Ser  
 195 200 205  
 5 Asn Leu Gly Asn Ile His Ala Ser Asn Asn Ile Met Phe Lys Leu Asp  
 210 215 220  
 Gly Cys Arg Asp Thr His Arg Tyr Ile Trp Ile Lys Tyr Phe Asn Leu  
 225 230 235 240  
 10 Phe Asp Lys Glu Leu Asn Glu Lys Glu Ile Lys Asp Leu Tyr Asp Asn  
 245 250 255  
 Gln Ser Asn Ser Gly Ile Leu Lys Asp Phe Trp Gly Asp Tyr Leu Gln  
 260 265 270  
 15 Tyr Asp Lys Pro Tyr Tyr Met Leu Asn Leu Tyr Asp Pro Asn Lys Tyr  
 275 280 285  
 20 Val Asp Val Asn Asn Val Gly Ile Arg Gly Tyr Met Tyr Leu Lys Gly  
 290 295 300  
 Pro Arg Gly Ser Val Met Thr Thr Asn Ile Tyr Leu Asn Ser Ser Leu  
 305 310 315 320  
 25 Tyr Arg Gly Thr Lys Phe Ile Ile Lys Lys Tyr Ala Ser Gly Asn Lys  
 325 330 335  
 Asp Asn Ile Val Arg Asn Asn Asp Arg Val Tyr Ile Asn Val Val Val  
 340 345 350  
 30 Lys Asn Lys Glu Tyr Arg Leu Ala Thr Asn Ala Ser Gln Ala Gly Val  
 355 360 365  
 35 Glu Lys Ile Leu Ser Ala Leu Glu Ile Pro Asp Val Gly Asn Leu Ser  
 370 375 380  
 Gln Val Val Val Met Lys Ser Lys Asn Asp Gln Gly Ile Thr Asn Lys  
 385 390 395 400  
 40 Cys Lys Met Asn Leu Gln Asp Asn Asn Gly Asn Asp Ile Gly Phe Ile  
 405 410 415  
 Gly Phe His Gln Phe Asn Asn Ile Ala Lys Leu Val Ala Ser Asn Trp  
 420 425 430  
 45 Tyr Asn Arg Gln Ile Glu Arg Ser Ser Arg Thr Leu Gly Cys Ser Trp  
 435 440 445  
 50 Glu Phe Ile Pro Val Asp Asp Gly Trp Gly Glu Arg Pro Leu  
 450 455 460

## (2) INFORMATION FOR SEQ ID NO:27:

- 55 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 3891 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear  
 60 (ii) MOLECULE TYPE: DNA (genomic)

## (ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 1..3888

5

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

10	ATG CAA TTT GTT AAT AAA CAA TTT AAT TAT AAA GAT CCT GTA AAT GGT Met Gln Phe Val Asn Lys Gln Phe Asn Tyr Lys Asp Pro Val Asn Gly	48
15	GTT GAT ATT GCT TAT ATA AAA ATT CCA AAT GTA GGA CAA ATG CAA CCA Val Asp Ile Ala Tyr Ile Lys Ile Pro Asn Val Gly Gln Met Gln Pro	96
20	GTA AAA GCT TTT AAA ATT CAT AAT AAA ATA TGG GTT ATT CCA GAA AGA Val Lys Ala Phe Lys Ile His Asn Lys Ile Trp Val Ile Pro Glu Arg	144
25	GAT ACA TTT ACA AAT CCT GAA GAA GGA GAT TTA AAT CCA CCA CCA GAA Asp Thr Phe Thr Asn Pro Glu Gly Asp Leu Asn Pro Pro Pro Glu	192
30	GCA AAA CAA GTT CCA GTT TCA TAT TAT GAT TCA ACA TAT TTA AGT ACA Ala Lys Gln Val Pro Val Ser Tyr Tyr Asp Ser Thr Tyr Leu Ser Thr	240
35	GAT AAT GAA AAA GAT AAT TAT TTA AAG GGA GTT ACA AAA TTA TTT GAG Asp Asn Glu Lys Asp Asn Tyr Leu Lys Gly Val Thr Lys Leu Phe Glu	288
40	AGA ATT TAT TCA ACT GAT CTT GGA AGA ATG TTG TTA ACA TCA ATA GTA Arg Ile Tyr Ser Thr Asp Leu Gly Arg Met Leu Leu Thr Ser Ile Val	336
45	AGG GGA ATA CCA TTT TGG GGT GGA AGT ACA ATA GAT ACA GAA TTA AAA Arg Gly Ile Pro Phe Trp Gly Gly Ser Thr Ile Asp Thr Glu Leu Lys	384
50	GTT ATT GAT ACT AAT TGT ATT AAT GTG ATA CAA CCA GAT GGT AGT TAT Val Ile Asp Thr Asn Cys Ile Asn Val Ile Gln Pro Asp Gly Ser Tyr	432
55	AGA TCA GAA GAA CTT AAT CTA GTA ATA ATA GGA CCC TCA GCT GAT ATT Arg Ser Glu Glu Leu Asn Leu Val Ile Ile Gly Pro Ser Ala Asp Ile	480
60	ATA CAG TTT GAA TGT AAA AGC TTT GGA CAT GAA GTT TTG AAT CTT ACG Ile Gln Phe Glu Cys Lys Ser Phe Gly His Glu Val Leu Asn Leu Thr	528
65	CGA AAT GGT TAT GGC TCT ACT CAA TAC ATT AGA TTT AGC CCA GAT TTT Arg Asn Gly Tyr Gly Ser Thr Gln Tyr Ile Arg Phe Ser Pro Asp Phe	576
70	ACA TTT GGT TTT GAG GAG TCA CTT GAA GTT GAT ACA AAT CCT CTT TTA Thr Phe Gly Phe Glu Glu Ser Leu Glu Val Asp Thr Asn Pro Leu Leu	624
75	GGT GCA GGC AAA TTT GCT ACA GAT CCA GCA GTA ACA TTA GCA CAT GAA Gly Ala Gly Lys Phe Ala Thr Asp Pro Ala Val Thr Leu Ala His Glu	672
80	CTT ATA CAT GCT GGA CAT AGA TTA TAT GGA ATA GCA ATT AAT CCA AAT Leu Ile His Ala Gly His Arg Leu Tyr Gly Ile Ala Ile Asn Pro Asn	720
85	AGG GTT TTT AAA GTA AAT ACT AAT GCC TAT TAT GAA ATG AGT GGG TTA Arg Val Phe Lys Val Asn Thr Asn Ala Tyr Tyr Glu Met Ser Gly Leu	768

	GAA GTA AGC TTT GAG GAA CTT AGA ACA TTT GGG GGA CAT GAT GCA AAG	816
	Glu Val Ser Phe Glu Glu Leu Arg Thr Phe Gly Gly His Asp Ala Lys	
	260 265 270	
5	TTT ATA GAT AGT TTA CAG GAA AAC GAA TTT CGT CTA TAT TAT TAT AAT	864
	Phe Ile Asp Ser Leu Gln Glu Asn Glu Phe Arg Leu Tyr Tyr Tyr Asn	
	275 280 285	
10	AAG TTT AAA GAT ATA GCA AGT ACA CTT AAT AAA GCT AAA TCA ATA GTA	912
	Lys Phe Lys Asp Ile Ala Ser Thr Leu Asn Lys Ala Lys Ser Ile Val	
	290 295 300	
15	GGT ACT ACT GCT TCA TTA CAG TAT ATG AAA AAT GTT TTT AAA GAG AAA	960
	Gly Thr Thr Ala Ser Leu Gln Tyr Met Lys Asn Val Phe Lys Glu Lys	
	305 310 315 320	
20	TAT CTC CTA TCT GAA GAT ACA TCT GGA AAA TTT TCG GTA GAT AAA TTA	1008
	Tyr Leu Leu Ser Glu Asp Thr Ser Gly Lys Phe Ser Val Asp Lys Leu	
	325 330 335	
	AAA TTT GAT AAG TTA TAC AAA ATG TTA ACA GAG ATT TAC ACA GAG GAT	1056
	Lys Phe Asp Lys Leu Tyr Lys Met Leu Thr Glu Ile Tyr Thr Glu Asp	
	340 345 350	
25	AAT TTT GTT AAG TTT TTT AAA GTA CTT AAC AGA AAA ACA TAT TTG AAT	1104
	Asn Phe Val Lys Phe Phe Lys Val Leu Asn Arg Lys Thr Tyr Leu Asn	
	355 360 365	
30	TTT GAT AAA GCC GTA TTT AAG ATA AAT ATA GTA CCT AAG GTA AAT TAC	1152
	Phe Asp Lys Ala Val Phe Lys Ile Asn Ile Val Pro Lys Val Asn Tyr	
	370 375 380	
35	ACA ATA TAT GAT GGA TTT AAT TTA AGA AAT ACA AAT TTA GCA GCA AAC	1200
	Thr Ile Tyr Asp Gly Phe Asn Leu Arg Asn Thr Asn Leu Ala Ala Asn	
	385 390 395 400	
40	TTT AAT GGT CAA AAT ACA GAA ATT AAT AAT ATG AAT TTT ACT AAA CTA	1248
	Phe Asn Gly Gln Asn Thr Glu Ile Asn Asn Met Asn Phe Thr Lys Leu	
	405 410 415	
	AAA AAT TTT ACT GGA TTG TTT GAA TTT TAT AAG TTG CTA TGT GTA AGA	1296
	Lys Asn Phe Thr Gly Leu Phe Glu Phe Tyr Lys Leu Leu Cys Val Arg	
	420 425 430	
45	GGG ATA ATA ACT TCT AAA ACT AAA TCA TTA GAT AAA GGA TAC AAT AAG	1344
	Gly Ile Ile Thr Ser Lys Thr Lys Ser Leu Asp Lys Gly Tyr Asn Lys	
	435 440 445	
50	GCA TTA AAT GAT TTA TGT ATC AAA GTT AAT AAT TGG GAC TTG TTT TTT	1392
	Ala Leu Asn Asp Leu Cys Ile Lys Val Asn Asn Trp Asp Leu Phe Phe	
	450 455 460	
55	AGT CCT TCA GAA GAT AAT TTT ACT AAT GAT CTA AAT AAA GGA GAA GAA	1440
	Ser Pro Ser Glu Asp Asn Phe Thr Asn Asp Leu Asn Lys Gly Glu Glu	
	465 470 475 480	
60	ATT ACA TCT GAT ACT AAT ATA GAA GCA GCA GAA GAA AAT ATT AGT TTA	1488
	Ile Thr Ser Asp Thr Asn Ile Glu Ala Ala Glu Glu Asn Ile Ser Leu	
	485 490 495	
	GAT TTA ATA CAA CAA TAT TAT TTA ACC TTT AAT TTT GAT AAT GAA CCT	1536
	Asp Leu Ile Gln Gln Tyr Tyr Leu Thr Phe Asn Phe Asp Asn Glu Pro	
	500 505 510	
65	GAA AAT ATT TCA ATA GAA AAT CTT TCA AGT GAC ATT ATA GGC CAA TTA	1584
	Glu Asn Ile Ser Ile Glu Asn Leu Ser Ser Asp Ile Ile Gly Gln Leu	
	515 520 525	
70	GAA CTT ATG CCT AAT ATA GAA AGA TTT CCT AAT GGA AAA AAG TAT GAG	1632
	Glu Leu Met Pro Asn Ile Glu Arg Phe Pro Asn Gly Lys Lys Tyr Glu	

	530	535	540	
5	TTA GAT AAA TAT ACT ATG TTC CAT TAT CTT CGT GCT CAA GAA TTT GAA Leu Asp Lys Tyr Thr Met Phe His Tyr Leu Arg Ala Gln Glu Phe Glu 545 550 555 560			1680
10	CAT GGT AAA TCT AGG ATT GCT TTA ACA AAT TCT GTT AAC GAA GCA TTA His Gly Lys Ser Arg Ile Ala Leu Thr Asn Ser Val Asn Glu Ala Leu 565 570 575			1728
15	TTA AAT CCT AGT CGT GTT TAT ACA TTT TTT TCT TCA GAC TAT GTA AAG Leu Asn Pro Ser Arg Val Tyr Thr Phe Phe Ser Ser Asp Tyr Val Lys 580 585 590			1776
20	AAA GTT AAT AAA GCT ACG GAG GCA GCT ATG TTT TTA GGC TGG GTA GAA Lys Val Asn Lys Ala Thr Glu Ala Met Phe Leu Gly Trp Val Glu 595 600 605			1824
25	CAA TTA GTA TAT GAT TTT ACC GAT GAA ACT AGC GAA GTA AGT ACT ACG Gln Leu Val Tyr Asp Phe Thr Asp Glu Thr Ser Glu Val Ser Thr Thr 610 615 620			1872
30	GAT AAA ATT GCG GAT ATA ACT ATA ATT ATT CCA TAT ATA GGA CCT GCT Asp Lys Ile Ala Asp Ile Thr Ile Ile Ile Pro Tyr Ile Gly Pro Ala 625 630 635 640			1920
35	TTA AAT ATA GGT AAT ATG TTA TAT AAA GAT TTT GTA GGT GCT TTA Leu Asn Ile Gly Asn Met Leu Tyr Lys Asp Phe Val Gly Ala Leu 645 650 655			1968
40	ATA TTT TCA GGA GCT GTT ATT CTG TTA GAA TTT ATA CCA GAG ATT GCA Ile Phe Ser Gly Ala Val Ile Leu Leu Glu Phe Ile Pro Glu Ile Ala 660 665 670			2016
45	ATA CCT GTA TTA GGT ACT TTT GCA CTT GTA TCA TAT ATT GCG AAT AAG Ile Pro Val Leu Gly Thr Phe Ala Leu Val Ser Tyr Ile Ala Asn Lys 675 680 685			2064
50	GTT CTA ACC GTT CAA ACA ATA GAT AAT GCT TTA AGT AAA AGA AAT GAA Val Leu Thr Val Gln Thr Ile Asp Asn Ala Leu Ser Lys Arg Asn Glu 690 695 700			2112
55	AAA TGG GAT GAG GTC TAT AAA TAT ATA GTA ACA AAT TGG TTA GCA AAG Lys Trp Asp Glu Val Tyr Lys Tyr Ile Val Thr Asn Trp Leu Ala Lys 705 710 715 720			2160
60	GTT AAT ACA CAG ATT GAT CTA ATA AGA AAA AAA ATG AAA GAA GCT TTA Val Asn Thr Gln Ile Asp Leu Ile Arg Lys Lys Met Lys Glu Ala Leu 725 730 735			2208
65	GAA AAT CAA GCA GAA GCA ACA AAG GCT ATA ATA AAC TAT CAG TAT AAT Glu Asn Gln Ala Glu Ala Thr Lys Ala Ile Ile Asn Tyr Gln Tyr Asn 740 745 750			2256
70	CAA TAT ACT GAG GAA GAG AAA AAT AAT ATT AAT TTT AAT ATT GAT GAT Gln Tyr Thr Glu Glu Glu Lys Asn Asn Ile Asn Phe Asn Ile Asp Asp 755 760 765			2304
75	TTA AGT TCG AAA CTT AAT GAG TCT ATA AAT AAA GCT ATG ATT AAT ATA Leu Ser Ser Lys Leu Asn Glu Ser Ile Asn Lys Ala Met Ile Asn Ile 770 775 780			2352
80	AAT AAA TTT TTG AAT CAA TGC TCT GTT TCA TAT TTA ATG AAT TCT ATG Asn Lys Phe Leu Asn Gln Cys Ser Val Ser Tyr Leu Met Asn Ser Met 785 790 795 800			2400
85	ATC CCT TAT GGT GTT AAA CGG TTA GAA GAT TTT GAT GCT AGT CTT AAA Ile Pro Tyr Gly Val Lys Arg Leu Glu Asp Phe Asp Ala Ser Leu Lys 805 810 815			2448

	GAT GCA TTA TTA AAG TAT ATA TAT GAT AAT AGA GGA ACT TTA ATT GGT	2496
	Asp Ala Leu Leu Lys Tyr Ile Tyr Asp Asn Arg Gly Thr Leu Ile Gly	
	820 825 830	
5	CAA GTA GAT AGA TTA AAA GAT AAA GTT AAT AAT ACA CTT AGT ACA GAT	2544
	Gln Val Asp Arg Leu Lys Asp Lys Val Asn Asn Thr Leu Ser Thr Asp	
	835 840 845	
10	ATA CCT TTT CAG CTT TCC AAA TAC GTA GAT AAT CAA AGA TTA TTA TCT	2592
	Ile Pro Phe Gln Leu Ser Lys Tyr Val Asp Asn Gln Arg Leu Leu Ser	
	850 855 860	
15	ACA TTT ACT GAA TAT ATT AAG AAT ATT ATT AAT ACT TCT ATA TTG AAT	2640
	Thr Phe Thr Glu Tyr Ile Lys Asn Ile Ile Asn Thr Ser Ile Leu Asn	
	865 870 875 880	
20	TTA AGA TAT GAA AGT AAT CAT TTA ATA GAC TTA TCT AGG TAT GCA TCA	2688
	Leu Arg Tyr Glu Ser Asn His Leu Ile Asp Leu Ser Arg Tyr Ala Ser	
	885 890 895	
	AAA ATA AAT ATT GGT AGT AAA GTA AAT TTT GAT CCA ATA GAT AAA AAT	2736
	Lys Ile Asn Ile Gly Ser Lys Val Asn Phe Asp Pro Ile Asp Lys Asn	
	900 905 910	
25	CAA ATT CAA TTA TTT AAT TTA GAA AGT AGT AAA ATT GAG GTA ATT TTA	2784
	Gln Ile Gln Leu Phe Asn Leu Glu Ser Ser Lys Ile Glu Val Ile Leu	
	915 920 925	
30	AAA AAT GCT ATT GTA TAT AAT AGT ATG TAT GAA AAT TTT AGT ACT AGC	2832
	Lys Asn Ala Ile Val Tyr Asn Ser Met Tyr Glu Asn Phe Ser Thr Ser	
	930 935 940	
35	TTT TGG ATA AGA ATT CCT AAG TAT TTT AAC AGT ATA AGT CTA AAT AAT	2880
	Phe Trp Ile Arg Ile Pro Lys Tyr Phe Asn Ser Ile Ser Leu Asn Asn	
	945 950 955 960	
40	GAA TAT ACA ATA ATA AAT TGT ATG GAA AAT AAT TCA GGA TGG AAA GTA	2928
	Glu Tyr Thr Ile Ile Asn Cys Met Glu Asn Asn Ser Gly Trp Lys Val	
	965 970 975	
	TCA CTT AAT TAT GGT GAA ATA ATC TGG ACT TTA CAG GAT ACT CAG GAA	2976
	Ser Leu Asn Tyr Gly Glu Ile Ile Trp Thr Leu Gln Asp Thr Gln Glu	
	980 985 990	
45	ATA AAA CAA AGA GTA GTT TTT AAA TAC AGT CAA ATG ATT AAT ATA TCA	3024
	Ile Lys Gln Arg Val Val Phe Lys Tyr Ser Gln Met Ile Asn Ile Ser	
	995 1000 1005	
50	GAT TAT ATA AAC AGA TGG ATT TTT GTA ACT ATC ACT AAT AAT AGA TTA	3072
	Asp Tyr Ile Asn Arg Trp Ile Phe Val Thr Ile Thr Asn Asn Arg Leu	
	1010 1015 1020	
55	AAT AAC TCT AAA ATT TAT ATA AAT GGA AGA TTA ATA GAT CAA AAA CCA	3120
	Asn Asn Ser Lys Ile Tyr Ile Asn Gly Arg Leu Ile Asp Gln Lys Pro	
	1025 1030 1035 1040	
60	ATT TCA AAT TTA GGT AAT ATT CAT GCT AGT AAT AAT ATA ATG TTT AAA	3168
	Ile Ser Asn Leu Gly Asn Ile His Ala Ser Asn Asn Ile Met Phe Lys	
	1045 1050 1055	
	TTA GAT GGT TGT AGA GAT ACA CAT AGA TAT ATT TGG ATA AAA TAT TTT	3216
	Leu Asp Gly Cys Arg Asp Thr His Arg Tyr Ile Trp Ile Lys Tyr Phe	
	1060 1065 1070	
65	AAT CTT TTT GAT AAG GAA TTA AAT GAA AAA GAA ATC AAA GAT TTA TAT	3264
	Asn Leu Phe Asp Lys Glu Leu Asn Glu Lys Glu Ile Lys Asp Leu Tyr	
	1075 1080 1085	
70	GAT AAT CAA TCA AAT TCA GGT ATT TTA AAA GAC TTT TGG GGT GAT TAT	3312
	Asp Asn Gln Ser Asn Ser Gly Ile Leu Lys Asp Phe Trp Gly Asp Tyr	

	1090	1095	1100	
5	TTA CAA TAT GAT AAA CCA TAC TAT ATG TTA AAT TTA TAT GAT CCA AAT Leu Gln Tyr Asp Lys Pro Tyr Tyr Met Leu Asn Leu Tyr Asp Pro Asn 1105 1110 1115 1120			3360
10	AAA TAT GTC GAT GTA AAT AAT GTA GGT ATT AGA GGT TAT ATG TAT CTT Lys Tyr Val Asp Val Asn Asn Val Gly Ile Arg Gly Tyr Met Tyr Leu 1125 1130 1135			3408
15	AAA GGG CCT AGA GGT AGC GTA ATG ACT ACA AAC ATT TAT TTA AAT TCA Lys Gly Pro Arg Gly Ser Val Met Thr Thr Asn Ile Tyr Leu Asn Ser 1140 1145 1150			3456
20	AGT TTG TAT AGG GGG ACA AAA TTT ATT ATA AAA AAA TAT GCT TCT GGA Ser Leu Tyr Arg Gly Thr Lys Phe Ile Ile Lys Lys Tyr Ala Ser Gly 1155 1160 1165			3504
25	AAT AAA GAT AAT ATT GTT AGA AAT AAT GAT CGT GTA TAT ATT AAT GTA Asn Lys Asp Asn Ile Val Arg Asn Asn Asp Arg Val Tyr Ile Asn Val 1170 1175 1180			3552
30	GTA GTT AAA AAT AAA GAA TAT AGG TTA GCT ACT AAT GCA TCA CAG GCA Val Val Lys Asn Lys Glu Tyr Arg Leu Ala Thr Asn Ala Ser Gln Ala 1185 1190 1195 1200			3600
35	GGC GTA GAA AAA ATA CTA AGT GCA TTA GAA ATA CCT GAT GTA GGA AAT Gly Val Glu Lys Ile Leu Ser Ala Leu Glu Ile Pro Asp Val Gly Asn 1205 1210 1215			3648
40	CTA AGT CAA GTA GTA GTA ATG AAG TCA AAA AAT GAT CAA GGA ATA ACA Leu Ser Gln Val Val Met Lys Ser Lys Asn Asp Gln Gly Ile Thr 1220 1225 1230			3696
45	AAT AAA TGC AAA ATG AAT TTA CAA GAT AAT AAT GGG AAT GAT ATA GGC Asn Lys Cys Lys Met Asn Leu Gln Asp Asn Asn Gly Asn Asp Ile Gly 1235 1240 1245			3744
50	TTT ATA GGA TTT CAT CAG TTT AAT AAT ATA GCT AAA CTA GTA GCA AGT Phe Ile Gly Phe His Gln Phe Asn Asn Ile Ala Lys Leu Val Ala Ser 1250 1255 1260			3792
55	AAT TGG TAT AAT AGA CAA ATA GAA AGA TCT AGT AGG ACT TTG GGT TGC Asn Trp Tyr Asn Arg Gln Ile Glu Arg Ser Ser Arg Thr Leu Gly Cys 1265 1270 1275 1280			3840
60	TCA TGG GAA TTT ATT CCT GTA GAT GAT GGA TGG GGA GAA AGG CCA CTG Ser Trp Glu Phe Ile Pro Val Asp Asp Gly Trp Gly Glu Arg Pro Leu 1285 1290 1295			3888
65	TAA			3891

## (2) INFORMATION FOR SEQ ID NO:28:

- 55 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 1296 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear
- 60 (ii) MOLECULE TYPE: protein
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:
- 65 Met Gln Phe Val Asn Lys Gln Phe Asn Tyr Lys Asp Pro Val Asn Gly  
 1 5 10 15
- Val Asp Ile Ala Tyr Ile Lys Ile Pro Asn Val Gly Gln Met Gln Pro  
 20 25 30
- 70 Val Lys Ala Phe Lys Ile His Asn Lys Ile Trp Val Ile Pro Glu Arg

	35	40	45
5	Asp Thr Phe Thr Asn Pro Glu Glu Gly Asp Leu Asn Pro Pro Pro Glu 50 55 60		
	Ala Lys Gln Val Pro Val Ser Tyr Tyr Asp Ser Thr Tyr Leu Ser Thr 65 70 75 80		
10	Asp Asn Glu Lys Asp Asn Tyr Leu Lys Gly Val Thr Lys Leu Phe Glu 85 90 95		
	Arg Ile Tyr Ser Thr Asp Leu Gly Arg Met Leu Leu Thr Ser Ile Val 100 105 110		
15	Arg Gly Ile Pro Phe Trp Gly Gly Ser Thr Ile Asp Thr Glu Leu Lys 115 120 125		
	Val Ile Asp Thr Asn Cys Ile Asn Val Ile Gln Pro Asp Gly Ser Tyr 130 135 140		
20	Arg Ser Glu Glu Leu Asn Leu Val Ile Ile Gly Pro Ser Ala Asp Ile 145 150 155 160		
	Ile Gln Phe Glu Cys Lys Ser Phe Gly His Glu Val Leu Asn Leu Thr 165 170 175		
25	Arg Asn Gly Tyr Gly Ser Thr Gln Tyr Ile Arg Phe Ser Pro Asp Phe 180 185 190		
30	Thr Phe Gly Phe Glu Glu Ser Leu Glu Val Asp Thr Asn Pro Leu Leu 195 200 205		
	Gly Ala Gly Lys Phe Ala Thr Asp Pro Ala Val Thr Leu Ala His Glu 210 215 220		
35	Leu Ile His Ala Gly His Arg Leu Tyr Gly Ile Ala Ile Asn Pro Asn 225 230 235 240		
	Arg Val Phe Lys Val Asn Thr Asn Ala Tyr Tyr Glu Met Ser Gly Leu 245 250 255		
40	Glu Val Ser Phe Glu Glu Leu Arg Thr Phe Gly Gly His Asp Ala Lys 260 265 270		
45	Phe Ile Asp Ser Leu Gln Glu Asn Glu Phe Arg Leu Tyr Tyr Tyr Asn 275 280 285		
	Lys Phe Lys Asp Ile Ala Ser Thr Leu Asn Lys Ala Lys Ser Ile Val 290 295 300		
50	Gly Thr Thr Ala Ser Leu Gln Tyr Met Lys Asn Val Phe Lys Glu Lys 305 310 315 320		
	Tyr Leu Leu Ser Glu Asp Thr Ser Gly Lys Phe Ser Val Asp Lys Leu 325 330 335		
55	Lys Phe Asp Lys Leu Tyr Lys Met Leu Thr Glu Ile Tyr Thr Glu Asp 340 345 350		
60	Asn Phe Val Lys Phe Phe Lys Val Leu Asn Arg Lys Thr Tyr Leu Asn 355 360 365		
	Phe Asp Lys Ala Val Phe Lys Ile Asn Ile Val Pro Lys Val Asn Tyr 370 375 380		
65	Thr Ile Tyr Asp Gly Phe Asn Leu Arg Asn Thr Asn Leu Ala Ala Asn 385 390 395 400		
70	Phe Asn Gly Gln Asn Thr Glu Ile Asn Asn Met Asn Phe Thr Lys Leu 405 410 415		



Lys Asn Phe Thr Gly Leu Phe Glu Phe Tyr Lys Leu Leu Cys Val Arg  
 420 425 430  
 5 Gly Ile Ile Thr Ser Lys Thr Lys Ser Leu Asp Lys Gly Tyr Asn Lys  
 435 440 445  
 Ala Leu Asn Asp Leu Cys Ile Lys Val Asn Asn Trp Asp Leu Phe Phe  
 450 455 460  
 10 Ser Pro Ser Glu Asp Asn Phe Thr Asn Asp Leu Asn Lys Gly Glu Glu  
 465 470 475 480  
 Ile Thr Ser Asp Thr Asn Ile Glu Ala Ala Glu Glu Asn Ile Ser Leu  
 485 490 495  
 15 Asp Leu Ile Gln Gln Tyr Tyr Leu Thr Phe Asn Phe Asp Asn Glu Pro  
 500 505 510  
 20 Glu Asn Ile Ser Ile Glu Asn Leu Ser Ser Asp Ile Ile Gly Gln Leu  
 515 520 525  
 Glu Leu Met Pro Asn Ile Glu Arg Phe Pro Asn Gly Lys Lys Tyr Glu  
 530 535 540  
 25 Leu Asp Lys Tyr Thr Met Phe His Tyr Leu Arg Ala Gln Glu Phe Glu  
 545 550 555 560  
 His Gly Lys Ser Arg Ile Ala Leu Thr Asn Ser Val Asn Glu Ala Leu  
 565 570 575  
 30 Leu Asn Pro Ser Arg Val Tyr Thr Phe Phe Ser Ser Asp Tyr Val Lys  
 580 585 590  
 35 Lys Val Asn Lys Ala Thr Glu Ala Ala Met Phe Leu Gly Trp Val Glu  
 595 600 605  
 Gln Leu Val Tyr Asp Phe Thr Asp Glu Thr Ser Glu Val Ser Thr Thr  
 610 615 620  
 40 Asp Lys Ile Ala Asp Ile Thr Ile Ile Ile Pro Tyr Ile Gly Pro Ala  
 625 630 635 640  
 Leu Asn Ile Gly Asn Met Leu Tyr Lys Asp Asp Phe Val Gly Ala Leu  
 645 650 655  
 45 Ile Phe Ser Gly Ala Val Ile Leu Leu Glu Phe Ile Pro Glu Ile Ala  
 660 665 670  
 50 Ile Pro Val Leu Gly Thr Phe Ala Leu Val Ser Tyr Ile Ala Asn Lys  
 675 680 685  
 Val Leu Thr Val Gln Thr Ile Asp Asn Ala Leu Ser Lys Arg Asn Glu  
 690 695 700  
 55 Lys Trp Asp Glu Val Tyr Lys Tyr Ile Val Thr Asn Trp Leu Ala Lys  
 705 710 715 720  
 Val Asn Thr Gln Ile Asp Leu Ile Arg Lys Lys Met Lys Glu Ala Leu  
 725 730 735  
 60 Glu Asn Gln Ala Glu Ala Thr Lys Ala Ile Ile Asn Tyr Gln Tyr Asn  
 740 745 750  
 65 Gln Tyr Thr Glu Glu Glu Lys Asn Asn Ile Asn Phe Asn Ile Asp Asp  
 755 760 765  
 Leu Ser Ser Lys Leu Asn Glu Ser Ile Asn Lys Ala Met Ile Asn Ile  
 770 775 780  
 70 Asn Lys Phe Leu Asn Gln Cys Ser Val Ser Tyr Leu Met Asn Ser Met

	785		790		795		800
	Ile Pro Tyr Gly	Val Lys Arg Leu Glu	Asp Phe Asp Ala Ser Leu Lys				
		805		810			815
5	Asp Ala Leu Leu	Lys Tyr Ile Tyr	Asp Asn Arg Gly Thr Leu Ile Gly				
		820	825			830	
10	Gln Val Asp Arg	Leu Lys Asp Lys Val Asn Asn Thr Leu Ser Thr Asp					
		835	840			845	
	Ile Pro Phe Gln	Leu Ser Lys Tyr Val Asp Asn Gln Arg Leu Leu Ser					
		850	855			860	
15	Thr Phe Thr Glu Tyr	Ile Lys Asn Ile Ile Asn Thr Ser Ile Leu Asn					
		865	870		875		880
	Leu Arg Tyr Glu	Ser Asn His Leu Ile Asp Leu Ser Arg Tyr Ala Ser					
		885		890			895
20	Lys Ile Asn Ile	Gly Ser Lys Val Asn Phe Asp Pro Ile Asp Lys Asn					
		900		905		910	
25	Gln Ile Gln Leu	Phe Asn Leu Glu Ser Ser Lys Ile Glu Val Ile Leu					
		915	920			925	
	Lys Asn Ala Ile	Val Tyr Asn Ser Met Tyr Glu Asn Phe Ser Thr Ser					
		930	935			940	
30	Phe Trp Ile Arg	Ile Pro Lys Tyr Phe Asn Ser Ile Ser Leu Asn Asn					
		945	950		955		960
	Glu Tyr Thr Ile	Ile Asn Cys Met Glu Asn Asn Ser Gly Trp Lys Val					
		965		970			975
35	Ser Leu Asn Tyr	Gly Glu Ile Ile Trp Thr Leu Gln Asp Thr Gln Glu					
		980		985			990
40	Ile Lys Gln Arg	Val Val Phe Lys Tyr Ser Gln Met Ile Asn Ile Ser					
		995	1000			1005	
	Asp Tyr Ile Asn	Arg Trp Ile Phe Val Thr Ile Thr Asn Asn Arg Leu					
		1010	1015			1020	
45	Asn Asn Ser Lys	Ile Tyr Ile Asn Gly Arg Leu Ile Asp Gln Lys Pro					
		1025	1030		1035		1040
	Ile Ser Asn Leu	Gly Asn Ile His Ala Ser Asn Asn Ile Met Phe Lys					
		1045		1050			1055
50	Leu Asp Gly Cys	Arg Asp Thr His Arg Tyr Ile Trp Ile Lys Tyr Phe					
		1060		1065			1070
55	Asn Leu Phe Asp	Lys Glu Leu Asn Glu Lys Glu Ile Lys Asp Leu Tyr					
		1075	1080			1085	
	Asp Asn Gln Ser	Asn Ser Gly Ile Leu Lys Asp Phe Trp Gly Asp Tyr					
		1090	1095			1100	
60	Leu Gln Tyr Asp	Lys Pro Tyr Tyr Met Leu Asn Leu Tyr Asp Pro Asn					
		1105	1110		1115		1120
	Lys Tyr Val Asp	Val Asn Asn Val Gly Ile Arg Gly Tyr Met Tyr Leu					
		1125		1130			1135
65	Lys Gly Pro Arg	Gly Ser Val Met Thr Thr Asn Ile Tyr Leu Asn Ser					
		1140		1145			1150
70	Ser Leu Tyr Arg	Gly Thr Lys Phe Ile Ile Lys Lys Tyr Ala Ser Gly					
		1155	1160			1165	

Asn Lys Asp Asn Ile Val Arg Asn Asn Asp Arg Val Tyr Ile Asn Val  
 1170 1175 1180  
 5 Val Val Lys Asn Lys Glu Tyr Arg Leu Ala Thr Asn Ala Ser Gln Ala  
 1185 1190 1195 1200  
 Gly Val Glu Lys Ile Leu Ser Ala Leu Glu Ile Pro Asp Val Gly Asn  
 1205 1210 1215  
 10 Leu Ser Gln Val Val Val Met Lys Ser Lys Asn Asp Gln Gly Ile Thr  
 1220 1225 1230  
 Asn Lys Cys Lys Met Asn Leu Gln Asp Asn Asn Gly Asn Asp Ile Gly  
 1235 1240 1245  
 15 Phe Ile Gly Phe His Gln Phe Asn Asn Ile Ala Lys Leu Val Ala Ser  
 1250 1255 1260  
 Asn Trp Tyr Asn Arg Gln Ile Glu Arg Ser Ser Arg Thr Leu Gly Cys  
 1265 1270 1275 1280  
 20 Ser Trp Glu Phe Ile Pro Val Asp Asp Gly Trp Gly Glu Arg Pro Leu  
 1285 1290 1295  
 25 (2) INFORMATION FOR SEQ ID NO:29:  
 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 30 base pairs  
 30 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear  
 (ii) MOLECULE TYPE: other nucleic acid  
 35 (A) DESCRIPTION: /desc = "DNA"  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:  
 CGCCATGGCT AGATTATTAT CTACATTTAC 30  
 40 (2) INFORMATION FOR SEQ ID NO:30:  
 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 26 base pairs  
 45 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear  
 (ii) MOLECULE TYPE: other nucleic acid  
 50 (A) DESCRIPTION: /desc = "DNA"  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:  
 GCAAGCTTCT TGACAGACTC ATGTAG 26  
 55 (2) INFORMATION FOR SEQ ID NO:31:  
 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 1546 base pairs  
 60 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear  
 (ii) MOLECULE TYPE: DNA (genomic)  
 65 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:  
 AGATCTCGAT CCCGCGAAAT TAATACGACT CACTATAGGG GAATTGTGAG CGGATAACAA 60  
 70 TTCCCCTCTA GAAATAATTT TGTTTAACTT TAAGAAGGAG ATATACCATG GGCCATCATC 120

ATCATCATCA TCATCATCAT CACAGCAGCG GCCATATCGA AGGTCGTCAT ATGGCTAGCA 180  
 TGGCTAGATT ATTATCTACA TTTACTGAAT ATATTAAGAA TATTATTAAT ACTTCTATAT 240  
 5 TGAATTAAAG ATATGAAAGT AATCATTTAA TAGACTTATC TAGGTATGCA TCAAAAATAA 300  
 ATATTGGTAG TAAAGTAAAT TTTGATCCAA TAGATAAAAA TCAAATTCAA TTATTTAATT 360  
 10 TAGAAAGTAG TAAAATTGAG GTAATTTTAA AAAATGCTAT TGTATATAAT AGTATGTATG 420  
 AAAATTTTAG TACTAGCTTT TGGATAAGAA TTCCTAAGTA TTTTAACAGT ATAAGTCTAA 480  
 ATAATGAATA TACAATAATA AATTGTATGG AAAATAATTC AGGATGGAAA GTATCACTTA 540  
 15 ATTATGGTGA AATAATCTGG ACTTTACAGG ATACTCAGGA AATAAAACAA AGAGTAGTTT 600  
 TTAAATACAG TCAAATGATT AATATATCAG ATTATATAAA CAGATGGATT TTTGTAACTA 660  
 20 TCACTAATAA TAGATTAAAT AACTCTAAAA TTTATATAAA TGGAAGATTA ATAGATCAAA 720  
 AACCAATTTT AAATTTAGGT AATATTCATG CTAGTAATAA TATAATGTTT AAATTAGATG 780  
 GTTGTAGAGA TACACATAGA TATATTTGGA TAAAATATTT TAATCTTTT GATAAGGAAT 840  
 25 TAAATGAAAA AGAAATCAAA GATTTTATATG ATAATCAATC AAATTCAGGT ATTTTAAAAG 900  
 ACTTTTGGGG TGATTATTTA CAATATGATA AACCATACTA TATGTAAAT TTATATGATC 960  
 30 CAAATAAATA TGTCGATGTA AATAATGTAG GTATTAGAGG TTATATGTAT CTAAAGGGC 1020  
 CTAGAGGTAG CGTAATGACT ACAAACATTT ATTTAAATTC AAGTTTGTAT AGGGGGACAA 1080  
 AATTTATTAT AAAAAAATAT GCTTCTGGAA ATAAAGATAA TATTGTTAGA AATAATGATC 1140  
 35 GTGTATATAT TAATGTAGTA GTTAAAAATA AAGAATATAG GTTAGCTACT AATGCATCAC 1200  
 AGGCAGGCGT AGAAAAAATA CTAAGTGCAT TAGAAATACC TGATGTAGGA AATCTAAGTC 1260  
 40 AAGTAGTAGT AATGAAGTCA AAAAATGATC AAGGAATAAC AAATAAATGC AAAATGAATT 1320  
 TACAAGATAA TAATGGGAAT GATATAGGCT TTATAGGATT TCATCAGTTT AATAATATAG 1380  
 CTAAACTAGT AGCAAGTAAT TGGTATAATA GACAAATAGA AAGATCTAGT AGGACTTTGG 1440  
 45 GTTGCTCATG GGAATTTATT CCTGTAGATG ATGGATGGGG AGAAAGGCCA CTGTAATTAA 1500  
 TCTCAAACATA CATGAGTCTG TCAAGAAGCT TGCGCCCGCA CTCGAG 1546

50 (2) INFORMATION FOR SEQ ID NO:32:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 9 amino acids  
 (B) TYPE: amino acid  
 55 (C) STRANDEDNESS: not relevant  
 (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: peptide

60 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

Met His His His His His His Met Ala  
 1 5

65 (2) INFORMATION FOR SEQ ID NO:33:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 21 base pairs  
 (B) TYPE: nucleic acid  
 70 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

5 TATGCATCAC CATCACCATC A 21

(2) INFORMATION FOR SEQ ID NO:34:

10 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 23 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "DNA"

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:  
CATGTGATGG TGATGGTGAT GCA 23

(2) INFORMATION FOR SEQ ID NO:35:

25 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 1351 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: double  
(D) TOPOLOGY: linear

30 (ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "DNA"

35 (ix) FEATURE:  
(A) NAME/KEY: CDS  
(B) LOCATION: 1..1335

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

40 ATG CAT CAC CAT CAC CAT CAC ATG GCT CGT CTG CTG TCT ACC TTC ACT 48  
Met His His His His His His Met Ala Arg Leu Leu Ser Thr Phe Thr  
1 5 10 15

45 GAA TAC ATC AAG AAC ATC ATC AAT ACC TCC ATC CTG AAC CTG CGC TAC 96  
Glu Tyr Ile Lys Asn Ile Ile Asn Thr Ser Ile Leu Asn Leu Arg Tyr  
20 25 30

50 GAA TCC AAT CAC CTG ATC GAC CTG TCT CGC TAC GCT TCC AAA ATC AAC 144  
Glu Ser Asn His Leu Ile Asp Leu Ser Arg Tyr Ala Ser Lys Ile Asn  
35 40 45

55 ATC GGT TCT AAA GTT AAC TTC GAT CCG ATC GAC AAG AAT CAG ATC CAG 192  
Ile Gly Ser Lys Val Asn Phe Asp Pro Ile Asp Lys Asn Gln Ile Gln  
50 55 60

CTG TTC AAT CTG GAA TCT TCC AAA ATC GAA GTT ATC CTG AAG AAT GCT 240  
Leu Phe Asn Leu Glu Ser Ser Lys Ile Glu Val Ile Leu Lys Asn Ala  
65 70 75 80

60 ATC GTA TAC AAC TCT ATG TAC GAA AAC TTC TCC ACC TCC TTC TGG ATC 288  
Ile Val Tyr Asn Ser Met Tyr Glu Asn Phe Ser Thr Ser Phe Trp Ile  
85 90 95

65 CGT ATC CCG AAA TAC TTC AAC TCC ATC TCT CTG AAC AAT GAA TAC ACC 336  
Arg Ile Pro Lys Tyr Phe Asn Ser Ile Ser Leu Asn Asn Glu Tyr Thr  
100 105 110

70 ATC ATC AAC TGC ATG GAA AAC AAT TCT GGT TGG AAA GTA TCT CTG AAC 384  
Ile Ile Asn Cys Met Glu Asn Asn Ser Gly Trp Lys Val Ser Leu Asn  
115 120 125

	TAC GGT GAA ATC ATC TGG ACT CTG CAG GAC ACT CAG GAA ATC AAA CAG Tyr Gly Glu Ile Ile Trp Thr Leu Gln Asp Thr Gln Glu Ile Lys Gln 130 135 140	432
5	CGT GTT GTA TTC AAA TAC TCT CAG ATG ATC AAC ATC TCT GAC TAC ATC Arg Val Val Phe Lys Tyr Ser Gln Met Ile Asn Ile Ser Asp Tyr Ile 145 150 155 160	480
10	AAT CGC TGG ATC TTC GTT ACC ATC ACC AAC AAT CGT CTG AAT AAC TCC Asn Arg Trp Ile Phe Val Thr Ile Thr Asn Asn Arg Leu Asn Asn Ser 165 170 175	528
15	AAA ATC TAC ATC AAC GGC CGT CTG ATC GAC CAG AAA CCG ATC TCC AAT Lys Ile Tyr Ile Asn Gly Arg Leu Ile Asp Gln Lys Pro Ile Ser Asn 180 185 190	576
20	CTG GGT AAC ATC CAC GCT TCT AAT AAC ATC ATG TTC AAA CTG GAC GGT Leu Gly Asn Ile His Ala Ser Asn Asn Ile Met Phe Lys Leu Asp Gly 195 200 205	624
	TGT CGT GAC ACT CAC CGC TAC ATC TGG ATC AAA TAC TTC AAT CTG TTC Cys Arg Asp Thr His Arg Tyr Ile Trp Ile Lys Tyr Phe Asn Leu Phe 210 215 220	672
25	GAC AAA GAA CTG AAC GAA AAA GAA ATC AAA GAC CTG TAC GAC AAC CAG Asp Lys Glu Leu Asn Glu Lys Glu Ile Lys Asp Leu Tyr Asp Asn Gln 225 230 235 240	720
30	TCC AAT TCT GGT ATC CTG AAA GAC TTC TGG GGT GAC TAC CTG CAG TAC Ser Asn Ser Gly Ile Leu Lys Asp Phe Trp Gly Asp Tyr Leu Gln Tyr 245 250 255	768
35	GAC AAA CCG TAC TAC ATG CTG AAT CTG TAC GAT CCG AAC AAA TAC GTT Asp Lys Pro Tyr Tyr Met Leu Asn Leu Tyr Asp Pro Asn Lys Tyr Val 260 265 270	816
40	GAC GTC AAC AAT GTA GGT ATC CGC GGT TAC ATG TAC CTG AAA GGT CCG Asp Val Asn Asn Val Gly Ile Arg Gly Tyr Met Tyr Leu Lys Gly Pro 275 280 285	864
	CGT GGT TCT GTT ATG ACT ACC AAC ATC TAC CTG AAC TCT TCC CTG TAC Arg Gly Ser Val Met Thr Thr Asn Ile Tyr Leu Asn Ser Ser Leu Tyr 290 295 300	912
45	CGT GGT ACC AAA TTC ATC ATC AAG AAA TAC GCG TCT GGT AAC AAG GAC Arg Gly Thr Lys Phe Ile Ile Lys Lys Tyr Ala Ser Gly Asn Lys Asp 305 310 315 320	960
50	AAT ATC GTT CGC AAC AAT GAT CGT GTA TAC ATC AAT GTT GTA GTT AAG Asn Ile Val Arg Asn Asn Asp Arg Val Tyr Ile Asn Val Val Val Lys 325 330 335	1008
55	AAC AAA GAA TAC CGT CTG GCT ACC AAT GCT TCT CAG GCT GGT GTA GAA Asn Lys Glu Tyr Arg Leu Ala Thr Asn Ala Ser Gln Ala Gly Val Glu 340 345 350	1056
	AAG ATC TTG TCT GCT CTG GAA ATC CCG GAC GTT GGT AAT CTG TCT CAG Lys Ile Leu Ser Ala Leu Glu Ile Pro Asp Val Gly Asn Leu Ser Gln 355 360 365	1104
60	GTA GTT GTA ATG AAA TCC AAG AAC GAC CAG GGT ATC ACT AAC AAA TGC Val Val Val Met Lys Ser Lys Asn Asp Gln Gly Ile Thr Asn Lys Cys 370 375 380	1152
65	AAA ATG AAT CTG CAG GAC AAC AAT GGT AAC GAT ATC GGT TTC ATC GGT Lys Met Asn Leu Gln Asp Asn Asn Gly Asn Asp Ile Gly Phe Ile Gly 385 390 395 400	1200
70	TTC CAC CAG TTC AAC AAT ATC GCT AAA CTG GTT GCT TCC AAC TGG TAC Phe His Gln Phe Asn Asn Ile Ala Lys Leu Val Ala Ser Asn Trp Tyr	1248

405 410 415  
 AAT CGT CAG ATC GAA CGT TCC TCT CGC ACT CTG GGT TGC TCT TGG GAG 1296  
 Asn Arg Gln Ile Glu Arg Ser Ser Arg Thr Leu Gly Cys Ser Trp Glu  
 420 425 430  
 TTC ATC CCG GTT GAT GAC GGT TGG GGT GAA CGT CCG CTG TAACCCGGGA 1345  
 Phe Ile Pro Val Asp Asp Gly Trp Gly Glu Arg Pro Leu  
 435 440 445  
 AAGCTT 1351  
 (2) INFORMATION FOR SEQ ID NO:36:  
 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 445 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear  
 (ii) MOLECULE TYPE: protein  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:  
 Met His His His His His His Met Ala Arg Leu Leu Ser Thr Phe Thr  
 1 5 10 15  
 Glu Tyr Ile Lys Asn Ile Ile Asn Thr Ser Ile Leu Asn Leu Arg Tyr  
 20 25 30  
 Glu Ser Asn His Leu Ile Asp Leu Ser Arg Tyr Ala Ser Lys Ile Asn  
 35 40 45  
 Ile Gly Ser Lys Val Asn Phe Asp Pro Ile Asp Lys Asn Gln Ile Gln  
 50 55 60  
 Leu Phe Asn Leu Glu Ser Ser Lys Ile Glu Val Ile Leu Lys Asn Ala  
 65 70 75 80  
 Ile Val Tyr Asn Ser Met Tyr Glu Asn Phe Ser Thr Ser Phe Trp Ile  
 85 90 95  
 Arg Ile Pro Lys Tyr Phe Asn Ser Ile Ser Leu Asn Asn Glu Tyr Thr  
 100 105 110  
 Ile Ile Asn Cys Met Glu Asn Asn Ser Gly Trp Lys Val Ser Leu Asn  
 115 120 125  
 Tyr Gly Glu Ile Ile Trp Thr Leu Gln Asp Thr Gln Glu Ile Lys Gln  
 130 135 140  
 Arg Val Val Phe Lys Tyr Ser Gln Met Ile Asn Ile Ser Asp Tyr Ile  
 145 150 155 160  
 Asn Arg Trp Ile Phe Val Thr Ile Thr Asn Asn Arg Leu Asn Asn Ser  
 165 170 175  
 Lys Ile Tyr Ile Asn Gly Arg Leu Ile Asp Gln Lys Pro Ile Ser Asn  
 180 185 190  
 Leu Gly Asn Ile His Ala Ser Asn Asn Ile Met Phe Lys Leu Asp Gly  
 195 200 205  
 Cys Arg Asp Thr His Arg Tyr Ile Trp Ile Lys Tyr Phe Asn Leu Phe  
 210 215 220  
 Asp Lys Glu Leu Asn Glu Lys Glu Ile Lys Asp Leu Tyr Asp Asn Gln  
 225 230 235 240  
 Ser Asn Ser Gly Ile Leu Lys Asp Phe Trp Gly Asp Tyr Leu Gln Tyr  
 245 250 255

Asp Lys Pro Tyr Tyr Met Leu Asn Leu Tyr Asp Pro Asn Lys Tyr Val  
 260 265 270  
 5 Asp Val Asn Asn Val Gly Ile Arg Gly Tyr Met Tyr Leu Lys Gly Pro  
 275 280 285  
 Arg Gly Ser Val Met Thr Thr Asn Ile Tyr Leu Asn Ser Ser Leu Tyr  
 290 295 300  
 10 Arg Gly Thr Lys Phe Ile Ile Lys Lys Tyr Ala Ser Gly Asn Lys Asp  
 305 310 315 320  
 Asn Ile Val Arg Asn Asn Asp Arg Val Tyr Ile Asn Val Val Val Lys  
 325 330 335  
 15 Asn Lys Glu Tyr Arg Leu Ala Thr Asn Ala Ser Gln Ala Gly Val Glu  
 340 345 350  
 20 Lys Ile Leu Ser Ala Leu Glu Ile Pro Asp Val Gly Asn Leu Ser Gln  
 355 360 365  
 Val Val Val Met Lys Ser Lys Asn Asp Gln Gly Ile Thr Asn Lys Cys  
 370 375 380  
 25 Lys Met Asn Leu Gln Asp Asn Asn Gly Asn Asp Ile Gly Phe Ile Gly  
 385 390 395 400  
 Phe His Gln Phe Asn Asn Ile Ala Lys Leu Val Ala Ser Asn Trp Tyr  
 405 410 415  
 30 Asn Arg Gln Ile Glu Arg Ser Ser Arg Thr Leu Gly Cys Ser Trp Glu  
 420 425 430  
 35 Phe Ile Pro Val Asp Asp Gly Trp Gly Glu Arg Pro Leu  
 435 440 445

(2) INFORMATION FOR SEQ ID NO:37:

- 40 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 27 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear  
 45 (ii) MOLECULE TYPE: other nucleic acid  
 (A) DESCRIPTION: /desc = "DNA"  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

50 CGCATATGAA TATTCGTCCA TTGCATG

27

(2) INFORMATION FOR SEQ ID NO:38:

- 55 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 27 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear  
 60 (ii) MOLECULE TYPE: other nucleic acid  
 (A) DESCRIPTION: /desc = "DNA"  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

65 GGAAGCTTGC AGGGCAATTA CATCATG

27

(2) INFORMATION FOR SEQ ID NO:39:

- 70 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 3876 base pairs



(B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear

5	(ii) MOLECULE TYPE: DNA (genomic)	
	(ix) FEATURE:	
	(A) NAME/KEY: CDS	
10	(B) LOCATION: 1..3873	
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:	
15	ATG CCA GTT ACA ATA AAT AAT TTT AAT TAT AAT GAT CCT ATT GAT AAT Met Pro Val Thr Ile Asn Asn Phe Asn Tyr Asn Asp Pro Ile Asp Asn	48
	1 5 10 15	
20	GAC AAT ATT ATT ATG ATG GAA CCT CCA TTT GCA AGG GGT ACG GGG AGA Asp Asn Ile Ile Met Met Glu Pro Pro Phe Ala Arg Gly Thr Gly Arg	96
	20 25 30	
25	TAT TAT AAA GCT TTT AAA ATC ACA GAT CGT ATT TGG ATA ATA CCC GAA Tyr Tyr Lys Ala Phe Lys Ile Thr Asp Arg Ile Trp Ile Ile Pro Glu	144
	35 40 45	
30	AGA TAT ACT TTT GGA TAT AAA CCT GAG GAT TTT AAT AAA AGT TCC GGT Arg Tyr Thr Phe Gly Tyr Lys Pro Glu Asp Phe Asn Lys Ser Ser Gly	192
	50 55 60	
35	ATT TTT AAT AGA GAT GTT TGT GAA TAT TAT GAT CCA GAT TAC TTA AAT Ile Phe Asn Arg Asp Val Cys Glu Tyr Tyr Asp Pro Asp Tyr Leu Asn	240
	65 70 75 80	
40	ACC AAT GAT AAA AAG AAT ATA TTT TTC CAA ACA TTG ATC AAG TTA TTT Thr Asn Asp Lys Lys Asn Ile Phe Phe Gln Thr Leu Ile Lys Leu Phe	288
	85 90 95	
45	AAT AGA ATC AAA TCA AAA CCA TTG GGT GAA AAG TTA TTA GAG ATG ATT Asn Arg Ile Lys Ser Lys Pro Leu Gly Glu Lys Leu Leu Glu Met Ile	336
	100 105 110	
50	ATA AAT GGT ATA CCT TAT CTT GGA GAT AGA CGT GTT CCA CTC GAA GAG Ile Asn Gly Ile Pro Tyr Leu Gly Asp Arg Arg Val Pro Leu Glu Glu	384
	115 120 125	
55	TTT AAC ACA AAC ATT GCT AGT GTA ACT GTT AAT AAA TTA ATT AGT AAT Phe Asn Thr Asn Ile Ala Ser Val Thr Val Asn Lys Leu Ile Ser Asn	432
	130 135 140	
60	CCA GGA GAA GTG GAG CGA AAA AAA GGT ATT TTC GCA AAT TTA ATA ATA Pro Gly Glu Val Glu Arg Lys Lys Gly Ile Phe Ala Asn Leu Ile Ile	480
	145 150 155 160	
65	TTT GGA CCT GGG CCA GTT TTA AAT GAA AAT GAG ACT ATA GAT ATA GGT Phe Gly Pro Gly Pro Val Leu Asn Glu Asn Glu Thr Ile Asp Ile Gly	528
	165 170 175	
70	ATA CAA AAT CAT TTT GCA TCA AGG GAA GGC TTT GGG GGT ATA ATG CAA Ile Gln Asn His Phe Ala Ser Arg Glu Gly Phe Gly Gly Ile Met Gln	576
	180 185 190	
75	ATG AAA TTT TGT CCA GAA TAT GTA AGC GTA TTT AAT AAT GTT CAA GAA Met Lys Phe Cys Pro Glu Tyr Val Ser Val Phe Asn Asn Val Gln Glu	624
	195 200 205	
80	AAC AAA GGC GCA AGT ATA TTT AAT AGA CGT GGA TAT TTT TCA GAT CCA Asn Lys Gly Ala Ser Ile Phe Asn Arg Arg Gly Tyr Phe Ser Asp Pro	672
	210 215 220	
85	GCC TTG ATA TTA ATG CAT GAA CTT ATA CAT GTT TTG CAT GGA TTA TAT Ala Leu Ile Leu Met His Glu Leu Ile His Val Leu His Gly Leu Tyr	720

	225		230		235		240	
5	GGC ATT AAA GTA GAT GAT TTA CCA ATT GTA CCA AAT GAA AAA AAA TTT Gly Ile Lys Val Asp Asp Leu Pro Ile Val Pro Asn Glu Lys Lys Phe	245		250		255	768	
10	TTT ATG CAA TCT ACA GAT ACT ATA CAG GCA GAA GAA CTA TAT ACA TTT Phe Met Gln Ser Thr Asp Thr Ile Gln Ala Glu Glu Leu Tyr Thr Phe	260		265		270	816	
15	GGA GGA CAA GAT CCC AGC ATC ATA TCT CCT TCT ACA GAT AAA AGT ATC Gly Gly Gln Asp Pro Ser Ile Ile Ser Pro Ser Thr Asp Lys Ser Ile	275		280		285	864	
20	TAT GAT AAA GTT TTG CAA AAT TTT AGG GGG ATA GTT GAT AGA CTT AAC Tyr Asp Lys Val Leu Gln Asn Phe Arg Gly Ile Val Asp Arg Leu Asn	290		295		300	912	
25	AAG GTT TTA GTT TGC ATA TCA GAT CCT AAC ATT AAC ATT AAT ATA TAT Lys Val Leu Val Cys Ile Ser Asp Pro Asn Ile Asn Ile Asn Ile Tyr	305		310		315	960	
30	AAA AAT AAA TTT AAA GAT AAA TAT AAA TTC GTT GAA GAT TCT GAA GGA Lys Asn Lys Phe Lys Asp Lys Tyr Lys Phe Val Glu Asp Ser Glu Gly	325		330		335	1008	
35	AAA TAT AGT ATA GAT GTA GAA AGT TTC AAT AAA TTA TAT AAA AGC TTA Lys Tyr Ser Ile Asp Val Glu Ser Phe Asn Lys Leu Tyr Lys Ser Leu	340		345		350	1056	
40	ATG TTA GGT TTT ACA GAA ATT AAT ATA GCA GAA AAT TAT AAA ATA AAA Met Leu Gly Phe Thr Glu Ile Asn Ile Ala Glu Asn Tyr Lys Ile Lys	355		360		365	1104	
45	ACT AGA GCT TCT TAT TTT AGT GAT TCC TTA CCA CCA GTA AAA ATA AAA Thr Arg Ala Ser Tyr Phe Ser Asp Ser Leu Pro Pro Val Lys Ile Lys	370		375		380	1152	
50	AAT TTA TTA GAT AAT GAA ATC TAT ACT ATA GAG GAA GGG TTT AAT ATA Asn Leu Leu Asp Asn Glu Ile Tyr Thr Ile Glu Glu Gly Phe Asn Ile	385		390		395	1200	
55	TCT GAT AAA AAT ATG GGA AAA GAA TAT AGG GGT CAG AAT AAA GCT ATA Ser Asp Lys Asn Met Gly Lys Glu Tyr Arg Gly Gln Asn Lys Ala Ile	405		410		415	1248	
60	AAT AAA CAA GCT TAT GAA GAA ATC AGC AAG GAG CAT TTG GCT GTA TAT Asn Lys Gln Ala Tyr Glu Glu Ile Ser Lys Glu His Leu Ala Val Tyr	420		425		430	1296	
65	AAG ATA CAA ATG TGT AAA AGT GTT AAA GTT CCA GGA ATA TGT ATT GAT Lys Ile Gln Met Cys Lys Ser Val Lys Val Pro Gly Ile Cys Ile Asp	435		440		445	1344	
70	GTC GAT AAT GAA AAT TTG TTC TTT ATA GCT GAT AAA AAT AGT TTT TCA Val Asp Asn Glu Asn Leu Phe Phe Ile Ala Asp Lys Asn Ser Phe Ser	450		455		460	1392	
	GAT GAT TTA TCT AAA AAT GAA AGA GTA GAA TAT AAT ACA CAG AAT AAT Asp Asp Leu Ser Lys Asn Glu Arg Val Glu Tyr Asn Thr Gln Asn Asn	465		470		475	1440	
	TAT ATA GGA AAT GAC TTT CCT ATA AAT GAA TTA ATT TTA GAT ACT GAT Tyr Ile Gly Asn Asp Phe Pro Ile Asn Glu Leu Ile Leu Asp Thr Asp	485		490		495	1488	
	TTA ATA AGT AAA ATA GAA TTA CCA AGT GAA AAT ACA GAA TCA CTT ACT Leu Ile Ser Lys Ile Glu Leu Pro Ser Glu Asn Thr Glu Ser Leu Thr	500		505		510	1536	

	GAT TTT AAT GTA GAT GTT CCA GTA TAT GAA AAA CAA CCC GCT ATA AAA Asp Phe Asn Val Asp Val Pro Val Tyr Glu Lys Gln Pro Ala Ile Lys	1584
	515 520 525	
5	AAA GTT TTT ACA GAT GAA AAT ACC ATC TTT CAA TAT TTA TAC TCT CAG Lys Val Phe Thr Asp Glu Asn Thr Ile Phe Gln Tyr Leu Tyr Ser Gln	1632
	530 535 540	
10	ACA TTT CCT CTA AAT ATA AGA GAT ATA AGT TTA ACA TCT TCA TTT GAT Thr Phe Pro Leu Asn Ile Arg Asp Ile Ser Leu Thr Ser Ser Phe Asp	1680
	545 550 555 560	
15	GAT GCA TTA TTA GTT TCT AGC AAA GTT TAT TCA TTT TTT TCT ATG GAT Asp Ala Leu Leu Val Ser Ser Lys Val Tyr Ser Phe Phe Ser Met Asp	1728
	565 570 575	
20	TAT ATT AAA ACT GCT AAT AAA GTA GTA GAA GCA GGA TTA TTT GCA GGT Tyr Ile Lys Thr Ala Asn Lys Val Val Glu Ala Gly Leu Phe Ala Gly	1776
	580 585 590	
25	TGG GTG AAA CAG ATA GTA GAT GAT TTT GTA ATC GAA GCT AAT AAA AGC Trp Val Lys Gln Ile Val Asp Asp Phe Val Ile Glu Ala Asn Lys Ser	1824
	595 600 605	
30	AGT ACT ATG GAT AAA ATT GCA GAT ATA TCT CTA ATT GTT CCT TAT ATA Ser Thr Met Asp Lys Ile Ala Asp Ile Ser Leu Ile Val Pro Tyr Ile	1872
	610 615 620	
35	GGA TTA GCT TTA AAT GTA GGA GAT GAA ACA GCT AAA GGA AAT TTT GAA Gly Leu Ala Leu Asn Val Gly Asp Glu Thr Ala Lys Gly Asn Phe Glu	1920
	625 630 635 640	
40	AGT GCT TTT GAG ATT GCA GGA TCC AGT ATT TTA CTA GAA TTT ATA CCA Ser Ala Phe Glu Ile Ala Gly Ser Ser Ile Leu Leu Glu Phe Ile Pro	1968
	645 650 655	
45	GAA CTT TTA ATA CCT GTA GTT GGA GTC TTT TTA TTA GAA TCA TAT ATT Glu Leu Leu Ile Pro Val Val Gly Val Phe Leu Leu Glu Ser Tyr Ile	2016
	660 665 670	
50	GAC AAT AAA AAT AAA ATT ATT AAA ACA ATA GAT AAT GCT TTA ACT AAA Asp Asn Lys Asn Lys Ile Ile Lys Thr Ile Asp Asn Ala Leu Thr Lys	2064
	675 680 685	
55	AGA GTG GAA AAA TGG ATT GAT ATG TAC GGA TTA ATA GTA GCG CAA TGG Arg Val Glu Lys Trp Ile Asp Met Tyr Gly Leu Ile Val Ala Gln Trp	2112
	690 695 700	
60	CTC TCA ACA GTT AAT ACT CAA TTT TAT ACA ATA AAA GAG GGA ATG TAT Leu Ser Thr Val Asn Thr Gln Phe Tyr Thr Ile Lys Glu Gly Met Tyr	2160
	705 710 715 720	
65	AAG GCT TTA AAT TAT CAA GCA CAA GCA TTG GAA GAA ATA ATA AAA TAC Lys Ala Leu Asn Tyr Gln Ala Gln Ala Leu Glu Glu Ile Ile Lys Tyr	2208
	725 730 735	
70	AAA TAT AAT ATA TAT TCT GAA GAG GAA AAG TCA AAT ATT AAC ATC AAT Lys Tyr Asn Ile Tyr Ser Glu Glu Glu Lys Ser Asn Ile Asn Ile Asn	2256
	740 745 750	
75	TTT AAT GAT ATA AAT TCT AAA CTT AAT GAT GGT ATT AAC CAA GCT ATG Phe Asn Asp Ile Asn Ser Lys Leu Asn Asp Gly Ile Asn Gln Ala Met	2304
	755 760 765	
80	GAT AAT ATA AAT GAT TTT ATA AAT GAA TGT TCT GTA TCA TAT TTA ATG Asp Asn Ile Asn Asp Phe Ile Asn Glu Cys Ser Val Ser Tyr Leu Met	2352
	770 775 780	
85	AAA AAA ATG ATT CCA TTA GCT GTA AAA AAA TTA CTA GAC TTT GAT AAT Lys Lys Met Ile Pro Leu Ala Val Lys Lys Leu Leu Asp Phe Asp Asn	2400

	785		790		795		800	
5	ACT CTC AAA AAA AAT TTA TTA AAT TAT ATA GAT GAA AAT AAA TTA TAT Thr Leu Lys Lys Asn Leu Leu Asn Tyr Ile Asp Glu Asn Lys Leu Tyr	2448	805	810	815			
10	TTA ATT GGA AGT GTA GAA GAT GAA AAA TCA AAA GTA GAT AAA TAC TTG Leu Ile Gly Ser Val Glu Asp Glu Lys Ser Lys Val Asp Lys Tyr Leu	2496	820	825	830			
15	AAA ACC ATT ATA CCA TTT GAT CTT TCA ACG TAT TCT AAT ATT GAA ATA Lys Thr Ile Ile Pro Phe Asp Leu Ser Thr Tyr Ser Asn Ile Glu Ile	2544	835	840	845			
20	CTA ATA AAA ATA TTT AAT AAA TAT AAT AGC GAA ATT TTA AAT AAT ATT Leu Ile Lys Ile Phe Asn Lys Tyr Asn Ser Glu Ile Leu Asn Asn Ile	2592	850	855	860			
25	ATC TTA AAT TTA AGA TAT AGA GAT AAT AAT TTA ATA GAT TTA TCA GGA Ile Leu Asn Leu Arg Tyr Arg Asp Asn Asn Ile Ile Asp Leu Ser Gly	2640	865	870	875			880
30	TAT GGA GCA AAG GTA GAG GTA TAT GAT GGG GTC AAG CTT AAT GAT AAA Tyr Gly Ala Lys Val Glu Val Tyr Asp Gly Val Lys Leu Asn Asp Lys	2688	885	890	895			
35	AAT CAA TTT AAA TTA ACT AGT TCA GCA GAT AGT AAG ATT AGA GTC ACT Asn Gln Phe Lys Leu Thr Ser Ser Ala Asp Ser Lys Ile Arg Val Thr	2736	900	905	910			
40	CAA AAT CAG AAT ATT ATA TTT AAT AGT ATG TTC CTT GAT TTT AGC GTT Gln Asn Gln Asn Ile Ile Phe Asn Ser Met Phe Leu Asp Phe Ser Val	2784	915	920	925			
45	AGC TTT TGG ATA AGG ATA CCT AAA TAT AGG AAT GAT GAT ATA CAA AAT Ser Phe Trp Ile Arg Ile Pro Lys Tyr Arg Asn Asp Asp Ile Gln Asn	2832	930	935	940			
50	TAT ATT CAT AAT GAA TAT ACG ATA ATT AAT TGT ATG AAA AAT AAT TCA Tyr Ile His Asn Glu Tyr Thr Ile Ile Asn Cys Met Lys Asn Asn Ser	2880	945	950	955			960
55	GCC TGG AAA ATA TCT ATT AGG GGT AAT AGG ATA ATA TGG ACC TTA ATT Gly Trp Lys Ile Ser Ile Arg Gly Asn Arg Ile Ile Trp Thr Leu Ile	2928	965	970	975			
60	GAT ATA AAT GGA AAA ACC AAA TCA GTA TTT TTT GAA TAT AAC ATA AGA Asp Ile Asn Gly Lys Thr Lys Ser Val Phe Phe Glu Tyr Asn Ile Arg	2976	980	985	990			
65	GAA GAT ATA TCA GAG TAT ATA AAT AGA TGG TTT TTT GTA ACT ATT ACT Glu Asp Ile Ser Glu Tyr Ile Asn Arg Trp Phe Phe Val Thr Ile Thr	3024	995	1000	1005			
70	AAT AAT TTG GAT AAT GCT AAA ATT TAT ATT AAT GGC ACG TTA GAA TCA Asn Asn Leu Asp Asn Ala Lys Ile Tyr Ile Asn Gly Thr Leu Glu Ser	3072	1010	1015	1020			
75	AAT ATG GAT ATT AAA GAT ATA GGA GAA GTT ATT GTT AAT GGT GAA ATA Asn Met Asp Ile Lys Asp Ile Gly Glu Val Ile Val Asn Gly Glu Ile	3120	1025	1030	1035			1040
80	ACA TTT AAA TTA GAT GGT GAT GTA GAT AGA ACA CAA TTT ATT TGG ATG Thr Phe Lys Leu Asp Gly Asp Val Asp Arg Thr Gln Phe Ile Trp Met	3168	1045	1050	1055			
85	AAA TAT TTT AGT ATT TTT AAT ACG CAA TTA AAT CAA TCA AAT ATT AAA Lys Tyr Phe Ser Ile Phe Asn Thr Gln Leu Asn Gln Ser Asn Ile Lys	3216	1060	1065	1070			

	GAG ATA TAT AAA ATT CAA TCA TAT AGC GAA TAC TTA AAA GAT TTT TGG Glu Ile Tyr Lys Ile Gln Ser Tyr Ser Glu Tyr Leu Lys Asp Phe Trp 1075 1080 1085	3264
5	GGA AAT CCT TTA ATG TAT AAT AAA GAA TAT TAT ATG TTT AAT GCG GGG Gly Asn Pro Leu Met Tyr Asn Lys Glu Tyr Tyr Met Phe Asn Ala Gly 1090 1095 1100	3312
10	AAT AAA AAT TCA TAT ATT AAA CTA GTG AAA GAT TCA TCT GTA GGT GAA Asn Lys Asn Ser Tyr Ile Lys Leu Val Lys Asp Ser Ser Val Gly Glu 1105 1110 1115 1120	3360
15	ATA TTA ATA CGT AGC AAA TAT AAT CAG AAT TCC AAT TAT ATA AAT TAT Ile Leu Ile Arg Ser Lys Tyr Asn Gln Asn Ser Asn Tyr Ile Asn Tyr 1125 1130 1135	3408
20	AGA AAT TTA TAT ATT GGA GAA AAA TTT ATT ATA AGA AGA GAG TCA AAT Arg Asn Leu Tyr Ile Gly Glu Lys Phe Ile Ile Arg Arg Glu Ser Asn 1140 1145 1150	3456
	TCT CAA TCT ATA AAT GAT GAT ATA GTT AGA AAA GAA GAT TAT ATA CAT Ser Gln Ser Ile Asn Asp Asp Ile Val Arg Lys Glu Asp Tyr Ile His 1155 1160 1165	3504
25	CTA GAT TTG GTA CTT CAC CAT GAA GAG TGG AGA GTA TAT GCC TAT AAA Leu Asp Leu Val Leu His His Glu Glu Trp Arg Val Tyr Ala Tyr Lys 1170 1175 1180	3552
30	TAT TTT AAG GAA CAG GAA GAA AAA TTG TTT TTA TCT ATT ATA AGT GAT Tyr Phe Lys Glu Gln Glu Glu Lys Leu Phe Leu Ser Ile Ile Ser Asp 1185 1190 1195 1200	3600
35	TCT AAT GAA TTT TAT AAG ACT ATA GAA ATA AAA GAA TAT GAT GAA CAG Ser Asn Glu Phe Tyr Lys Thr Ile Glu Ile Lys Glu Tyr Asp Glu Gln 1205 1210 1215	3648
40	CCA TCA TAT AGT TGT CAG TTG CTT TTT AAA AAA GAT GAA GAA AGT ACT Pro Ser Tyr Ser Cys Gln Leu Leu Phe Lys Lys Asp Glu Glu Ser Thr 1220 1225 1230	3696
	GAT GAT ATA GGA TTG ATT GGT ATT CAT CGT TTC TAC GAA TCT GGA GTT Asp Asp Ile Gly Leu Ile Gly Ile His Arg Phe Tyr Glu Ser Gly Val 1235 1240 1245	3744
45	TTA CGT AAA AAG TAT AAA GAT TAT TTT TGT ATA AGT AAA TGG TAC TTA Leu Arg Lys Lys Tyr Lys Asp Tyr Phe Cys Ile Ser Lys Trp Tyr Leu 1250 1255 1260	3792
50	AAA GAG GTA AAA AGG AAA CCA TAT AAG TCA AAT TTG GGA TGT AAT TGG Lys Glu Val Lys Arg Lys Pro Tyr Lys Ser Asn Leu Gly Cys Asn Trp 1265 1270 1275 1280	3840
55	CAG TTT ATT CCT AAA GAT GAA GGG TGG ACT GAA TAA Gln Phe Ile Pro Lys Asp Glu Gly Trp Thr Glu 1285 1290	3876

## (2) INFORMATION FOR SEQ ID NO:40:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 1291 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

Met Pro Val Thr Ile Asn Asn Phe Asn Tyr Asn Asp Pro Ile Asp Asn  
1 5 10 15

	Asp	Asn	Ile	Ile	Met	Met	Glu	Pro	Pro	Phe	Ala	Arg	Gly	Thr	Gly	Arg
				20					25						30	
5	Tyr	Tyr	Lys	Ala	Phe	Lys	Ile	Thr	Asp	Arg	Ile	Trp	Ile	Ile	Pro	Glu
			35					40					45			
	Arg	Tyr	Thr	Phe	Gly	Tyr	Lys	Pro	Glu	Asp	Phe	Asn	Lys	Ser	Ser	Gly
		50					55					60				
10	Ile	Phe	Asn	Arg	Asp	Val	Cys	Glu	Tyr	Tyr	Asp	Pro	Asp	Tyr	Leu	Asn
	65					70					75					80
	Thr	Asn	Asp	Lys	Lys	Asn	Ile	Phe	Phe	Gln	Thr	Leu	Ile	Lys	Leu	Phe
15					85					90					95	
	Asn	Arg	Ile	Lys	Ser	Lys	Pro	Leu	Gly	Glu	Lys	Leu	Leu	Glu	Met	Ile
				100					105					110		
20	Ile	Asn	Gly	Ile	Pro	Tyr	Leu	Gly	Asp	Arg	Arg	Val	Pro	Leu	Glu	Glu
			115					120					125			
	Phe	Asn	Thr	Asn	Ile	Ala	Ser	Val	Thr	Val	Asn	Lys	Leu	Ile	Ser	Asn
		130					135					140				
25	Pro	Gly	Glu	Val	Glu	Arg	Lys	Lys	Gly	Ile	Phe	Ala	Asn	Leu	Ile	Ile
	145					150					155					160
	Phe	Gly	Pro	Gly	Pro	Val	Leu	Asn	Glu	Asn	Glu	Thr	Ile	Asp	Ile	Gly
30					165					170					175	
	Ile	Gln	Asn	His	Phe	Ala	Ser	Arg	Glu	Gly	Phe	Gly	Gly	Ile	Met	Gln
				180					185					190		
35	Met	Lys	Phe	Cys	Pro	Glu	Tyr	Val	Ser	Val	Phe	Asn	Asn	Val	Gln	Glu
			195					200					205			
	Asn	Lys	Gly	Ala	Ser	Ile	Phe	Asn	Arg	Arg	Gly	Tyr	Phe	Ser	Asp	Pro
		210					215					220				
40	Ala	Leu	Ile	Leu	Met	His	Glu	Leu	Ile	His	Val	Leu	His	Gly	Leu	Tyr
	225					230					235					240
	Gly	Ile	Lys	Val	Asp	Asp	Leu	Pro	Ile	Val	Pro	Asn	Glu	Lys	Lys	Phe
45					245					250					255	
	Phe	Met	Gln	Ser	Thr	Asp	Thr	Ile	Gln	Ala	Glu	Glu	Leu	Tyr	Thr	Phe
				260					265					270		
50	Gly	Gly	Gln	Asp	Pro	Ser	Ile	Ile	Ser	Pro	Ser	Thr	Asp	Lys	Ser	Ile
			275					280					285			
	Tyr	Asp	Lys	Val	Leu	Gln	Asn	Phe	Arg	Gly	Ile	Val	Asp	Arg	Leu	Asn
		290					295					300				
55	Lys	Val	Leu	Val	Cys	Ile	Ser	Asp	Pro	Asn	Ile	Asn	Ile	Asn	Ile	Tyr
	305					310					315					320
	Lys	Asn	Lys	Phe	Lys	Asp	Lys	Tyr	Lys	Phe	Val	Glu	Asp	Ser	Glu	Gly
60					325					330					335	
	Lys	Tyr	Ser	Ile	Asp	Val	Glu	Ser	P							

	385		390		395		400									
	Ser	Asp	Lys	Asn	Met	Gly	Lys	Glu	Tyr	Arg	Gly	Gln	Asn	Lys	Ala	Ile
				405						410					415	
5	Asn	Lys	Gln	Ala	Tyr	Glu	Glu	Ile	Ser	Lys	Glu	His	Leu	Ala	Val	Tyr
			420						425					430		
	Lys	Ile	Gln	Met	Cys	Lys	Ser	Val	Lys	Val	Pro	Gly	Ile	Cys	Ile	Asp
10			435					440					445			
	Val	Asp	Asn	Glu	Asn	Leu	Phe	Phe	Ile	Ala	Asp	Lys	Asn	Ser	Phe	Ser
	450					455						460				
15	Asp	Asp	Leu	Ser	Lys	Asn	Glu	Arg	Val	Glu	Tyr	Asn	Thr	Gln	Asn	Asn
	465					470					475				480	
	Tyr	Ile	Gly	Asn	Asp	Phe	Pro	Ile	Asn	Glu	Leu	Ile	Leu	Asp	Thr	Asp
20				485					490					495		
	Leu	Ile	Ser	Lys	Ile	Glu	Leu	Pro	Ser	Glu	Asn	Thr	Glu	Ser	Leu	Thr
			500						505					510		
25	Asp	Phe	Asn	Val	Asp	Val	Pro	Val	Tyr	Glu	Lys	Gln	Pro	Ala	Ile	Lys
			515					520					525			
	Lys	Val	Phe	Thr	Asp	Glu	Asn	Thr	Ile	Phe	Gln	Tyr	Leu	Tyr	Ser	Gln
	530					535						540				
30	Thr	Phe	Pro	Leu	Asn	Ile	Arg	Asp	Ile	Ser	Leu	Thr	Ser	Ser	Phe	Asp
	545				550						555				560	
	Asp	Ala	Leu	Leu	Val	Ser	Ser	Lys	Val	Tyr	Ser	Phe	Phe	Ser	Met	Asp
35				565					570						575	
	Tyr	Ile	Lys	Thr	Ala	Asn	Lys	Val	Val	Glu	Ala	Gly	Leu	Phe	Ala	Gly
			580						585					590		
40	Trp	Val	Lys	Gln	Ile	Val	Asp	Asp	Phe	Val	Ile	Glu	Ala	Asn	Lys	Ser
		595						600					605			
	Ser	Thr	Met	Asp	Lys	Ile	Ala	Asp	Ile	Ser	Leu	Ile	Val	Pro	Tyr	Ile
	610					615						620				
45	Gly	Leu	Ala	Leu	Asn	Val	Gly	Asp	Glu	Thr	Ala	Lys	Gly	Asn	Phe	Glu
	625				630						635				640	
	Ser	Ala	Phe	Glu	Ile	Ala	Gly	Ser	Ser	Ile	Leu	Leu	Glu	Phe	Ile	Pro
50				645					650					655		
	Glu	Leu	Leu	Ile	Pro	Val	Val	Gly	Val	Phe	Leu	Leu	Glu	Ser	Tyr	Ile
			660					665					670			
55	Asp	Asn	Lys	Asn	Lys	Ile	Ile	Lys	Thr	Ile	Asp	Asn	Ala	Leu	Thr	Lys
		675						680					685			
	Arg	Val	Glu	Lys	Trp	Ile	Asp	Met	Tyr	Gly	Leu	Ile	Val	Ala	Gln	Trp
	690						695					700				
60	Leu	Ser	Thr	Val	Asn	Thr	Gln	Phe	Tyr	Thr	Ile	Lys	Glu	Gly	Met	Tyr
	705				710						715				720	
	Lys	Ala	Leu	Asn	Tyr	Gln	Ala	Gln	Ala	Leu	Glu	Glu	Ile	Ile	Lys	Tyr
65				725						730				735		
	Lys	Tyr	Asn	Ile	Tyr	Ser	Glu	Glu	Glu	Lys	Ser	Asn	Ile	Asn	Ile	Asn
			740					745					750			
70	Phe	Asn	Asp	Ile	Asn	Ser	Lys	Leu	Asn	Asp	Gly	Ile	Asn	Gln	Ala	Met
		755						760					765			

Asp Asn Ile Asn Asp Phe Ile Asn Glu Cys Ser Val Ser Tyr Leu Met  
 770 775 780  
 5 Lys Lys Met Ile Pro Leu Ala Val Lys Lys Leu Leu Asp Phe Asp Asn  
 785 790 795 800  
 Thr Leu Lys Lys Asn Leu Leu Asn Tyr Ile Asp Glu Asn Lys Leu Tyr  
 805 810 815  
 10 Leu Ile Gly Ser Val Glu Asp Glu Lys Ser Lys Val Asp Lys Tyr Leu  
 820 825 830  
 Lys Thr Ile Ile Pro Phe Asp Leu Ser Thr Tyr Ser Asn Ile Glu Ile  
 835 840 845  
 15 Leu Ile Lys Ile Phe Asn Lys Tyr Asn Ser Glu Ile Leu Asn Asn Ile  
 850 855 860  
 Ile Leu Asn Leu Arg Tyr Arg Asp Asn Asn Leu Ile Asp Leu Ser Gly  
 865 870 875 880  
 Tyr Gly Ala Lys Val Glu Val Tyr Asp Gly Val Lys Leu Asn Asp Lys  
 885 890 895  
 25 Asn Gln Phe Lys Leu Thr Ser Ser Ala Asp Ser Lys Ile Arg Val Thr  
 900 905 910  
 Gln Asn Gln Asn Ile Ile Phe Asn Ser Met Phe Leu Asp Phe Ser Val  
 915 920 925  
 30 Ser Phe Trp Ile Arg Ile Pro Lys Tyr Arg Asn Asp Asp Ile Gln Asn  
 930 935 940  
 Tyr Ile His Asn Glu Tyr Thr Ile Ile Asn Cys Met Lys Asn Asn Ser  
 945 950 955 960  
 Gly Trp Lys Ile Ser Ile Arg Gly Asn Arg Ile Ile Trp Thr Leu Ile  
 965 970 975  
 40 Asp Ile Asn Gly Lys Thr Lys Ser Val Phe Phe Glu Tyr Asn Ile Arg  
 980 985 990  
 Glu Asp Ile Ser Glu Tyr Ile Asn Arg Trp Phe Phe Val Thr Ile Thr  
 995 1000 1005  
 45 Asn Asn Leu Asp Asn Ala Lys Ile Tyr Ile Asn Gly Thr Leu Glu Ser  
 1010 1015 1020  
 Asn Met Asp Ile Lys Asp Ile Gly Glu Val Ile Val Asn Gly Glu Ile  
 1025 1030 1035 1040  
 Thr Phe Lys Leu Asp Gly Asp Val Asp Arg Thr Gln Phe Ile Trp Met  
 1045 1050 1055  
 55 Lys Tyr Phe Ser Ile Phe Asn Thr Gln Leu Asn Gln Ser Asn Ile Lys  
 1060 1065 1070  
 Glu Ile Tyr Lys Ile Gln Ser Tyr Ser Glu Tyr Leu Lys Asp Phe Trp  
 1075 1080 1085  
 60 Gly Asn Pro Leu Met Tyr Asn Lys Glu Tyr Tyr Met Phe Asn Ala Gly  
 1090 1095 1100  
 Asn Lys Asn Ser Tyr Ile Lys Leu Val Lys Asp Ser Ser Val Gly Glu  
 1105 1110 1115 1120  
 Ile Leu Ile Arg Ser Lys Tyr Asn Gln Asn Ser Asn Tyr Ile Asn Tyr  
 1125 1130 1135  
 70 Arg Asn Leu Tyr Ile Gly Glu Lys Phe Ile Ile Arg Arg Glu Ser Asn



1140 1145 1150  
 Ser Gln Ser Ile Asn Asp Asp Ile Val Arg Lys Glu Asp Tyr Ile His  
 1155 1160 1165  
 5 Leu Asp Leu Val Leu His His Glu Glu Trp Arg Val Tyr Ala Tyr Lys  
 1170 1175 1180  
 10 Tyr Phe Lys Glu Gln Glu Glu Lys Leu Phe Leu Ser Ile Ile Ser Asp  
 1185 1190 1195 1200  
 Ser Asn Glu Phe Tyr Lys Thr Ile Glu Ile Lys Glu Tyr Asp Glu Gln  
 1205 1210 1215  
 15 Pro Ser Tyr Ser Cys Gln Leu Leu Phe Lys Lys Asp Glu Glu Ser Thr  
 1220 1225 1230  
 20 Asp Asp Ile Gly Leu Ile Gly Ile His Arg Phe Tyr Glu Ser Gly Val  
 1235 1240 1245  
 Leu Arg Lys Lys Tyr Lys Asp Tyr Phe Cys Ile Ser Lys Trp Tyr Leu  
 1250 1255 1260  
 25 Lys Glu Val Lys Arg Lys Pro Tyr Lys Ser Asn Leu Gly Cys Asn Trp  
 1265 1270 1275 1280  
 Gln Phe Ile Pro Lys Asp Glu Gly Trp Thr Glu  
 1285 1290  
 30 (2) INFORMATION FOR SEQ ID NO:41:  
 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 3876 base pairs  
 (B) TYPE: nucleic acid  
 35 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear  
 (ii) MOLECULE TYPE: DNA (genomic)  
 40 (ix) FEATURE:  
 (A) NAME/KEY: CDS  
 (B) LOCATION: 1..3873  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:  
 45 ATG CCA GTT ACA ATA AAT AAT TTT AAT TAT AAT GAT CCT ATT GAT AAT 48  
 Met Pro Val Thr Ile Asn Asn Phe Asn Tyr Asn Asp Pro Ile Asp Asn  
 1 5 10 15  
 50 AAT AAT ATT ATT ATG ATG GAG CCT CCA TTT GCG AGA GGT ACG GGG AGA 96  
 Asn Asn Ile Ile Met Met Glu Pro Pro Phe Ala Arg Gly Thr Gly Arg  
 20 25 30  
 55 TAT TAT AAA GCT TTT AAA ATC ACA GAT CGT ATT TGG ATA ATA CCG GAA 144  
 Tyr Tyr Lys Ala Phe Lys Ile Thr Asp Arg Ile Trp Ile Ile Pro Glu  
 35 40 45  
 60 AGA TAT ACT TTT GGA TAT AAA CCT GAG GAT TTT AAT AAA AGT TCC GGT 192  
 Arg Tyr Thr Phe Gly Tyr Lys Pro Glu Asp Phe Asn Lys Ser Ser Gly  
 50 55 60  
 65 ATT TTT AAT AGA GAT GTT TGT GAA TAT TAT GAT CCA GAT TAC TTA AAT 240  
 Ile Phe Asn Arg Asp Val Cys Glu Tyr Tyr Asp Pro Asp Tyr Leu Asn  
 65 70 75 80  
 ACT AAT GAT AAA AAG AAT ATA TTT TTA CAA ACA ATG ATC AAG TTA TTT 288  
 Thr Asn Asp Lys Lys Asn Ile Phe Leu Gln Thr Met Ile Lys Leu Phe  
 85 90 95  
 70 AAT AGA ATC AAA TCA AAA CCA TTG GGT GAA AAG TTA TTA GAG ATG ATT 336

	Asn	Arg	Ile	Lys	Ser	Lys	Pro	Leu	Gly	Glu	Lys	Leu	Leu	Glu	Met	Ile	
				100					105					110			
5	ATA	AAT	GGT	ATA	CCT	TAT	CTT	GGA	GAT	AGA	CGT	GTT	CCA	CTC	GAA	GAG	384
	Ile	Asn	Gly	Ile	Pro	Tyr	Leu	Gly	Asp	Arg	Arg	Val	Pro	Leu	Glu	Glu	
			115					120					125				
10	TTT	AAC	ACA	AAC	ATT	GCT	AGT	GTA	ACT	GTT	AAT	AAA	TTA	ATC	AGT	AAT	432
	Phe	Asn	Thr	Asn	Ile	Ala	Ser	Val	Thr	Val	Asn	Lys	Leu	Ile	Ser	Asn	
			130				135					140					
15	CCA	GGA	GAA	GTG	GAG	CGA	AAA	AAA	GGT	ATT	TTC	GCA	AAT	TTA	ATA	ATA	480
	Pro	Gly	Glu	Val	Glu	Arg	Lys	Lys	Gly	Ile	Phe	Ala	Asn	Leu	Ile	Ile	
			145			150					155					160	
20	TTT	GGA	CCT	GGG	CCA	GTT	TTA	AAT	GAA	AAT	GAG	ACT	ATA	GAT	ATA	GGT	528
	Phe	Gly	Pro	Gly	Pro	Val	Leu	Asn	Glu	Asn	Glu	Thr	Ile	Asp	Ile	Gly	
					165					170					175		
25	ATA	CAA	AAT	CAT	TTT	GCA	TCA	AGG	GAA	GGC	TTC	GGG	GGT	ATA	ATG	CAA	576
	Ile	Gln	Asn	His	Phe	Ala	Ser	Arg	Glu	Gly	Phe	Gly	Gly	Ile	Met	Gln	
				180					185					190			
30	ATG	AAG	TTT	TGC	CCA	GAA	TAT	GTA	AGC	GTA	TTT	AAT	AAT	GTT	CAA	GAA	624
	Met	Lys	Phe	Cys	Pro	Glu	Tyr	Val	Ser	Val	Phe	Asn	Asn	Val	Gln	Glu	
			195					200					205				
35	AAC	AAA	GGC	GCA	AGT	ATA	TTT	AAT	AGA	CGT	GGA	TAT	TTT	TCA	GAT	CCA	672
	Asn	Lys	Gly	Ala	Ser	Ile	Phe	Asn	Arg	Arg	Gly	Tyr	Phe	Ser	Asp	Pro	
			210				215					220					
40	GCC	TTG	ATA	TTA	ATG	CAT	GAA	CTT	ATA	CAT	GTT	TTA	CAT	GGA	TTA	TAT	720
	Ala	Leu	Ile	Leu	Met	His	Glu	Leu	Ile	His	Val	Leu	His	Gly	Leu	Tyr	
			225			230					235					240	
45	GGC	ATT	AAA	GTA	GAT	GAT	TTA	CCA	ATT	GTA	CCA	AAT	GAA	AAA	AAA	TTT	768
	Gly	Ile	Lys	Val	Asp	Asp	Leu	Pro	Ile	Val	Pro	Asn	Glu	Lys	Lys	Phe	
					245					250					255		
50	TTT	ATG	CAA	TCT	ACA	GAT	GCT	ATA	CAG	GCA	GAA	GAA	CTA	TAT	ACA	TTT	816
	Phe	Met	Gln	Ser	Thr	Asp	Ala	Ile	Gln	Ala	Glu	Glu	Leu	Tyr	Thr	Phe	
				260					265					270			
55	GGA	GGA	CAA	GAT	CCC	AGC	ATC	ATA	ACT	CCT	TCT	ACG	GAT	AAA	AGT	ATC	864
	Gly	Gly	Gln	Asp	Pro	Ser	Ile	Ile	Thr	Pro	Ser	Thr	Asp	Lys	Ser	Ile	
			275					280					285				
60	TAT	GAT	AAA	GTT	TTG	CAA	AAT	TTT	AGA	GGG	ATA	GTT	GAT	AGA	CTT	AAC	912
	Tyr	Asp	Lys	Val	Leu	Gln	Asn	Phe	Arg	Gly	Ile	Val	Asp	Arg	Leu	Asn	
			290				295					300					
65	AAG	GTT	TTA	GTT	TGC	ATA	TCA	GAT	CCT	AAC	ATT	AAT	ATT	AAT	ATA	TAT	960
	Lys	Val	Leu	Val	Cys	Ile	Ser	Asp	Pro	Asn	Ile	Asn	Ile	Asn	Ile	Tyr	
			305			310					315					320	
70	AAA	AAT	AAA	TTT	AAA	GAT	AAA	TAT	AAA	TTC	GTT	GAA	GAT	TCT	GAG	GGA	1008
	Lys	Asn	Lys	Phe	Lys	Asp	Lys	Tyr	Lys	Phe	Val	Glu	Asp	Ser	Glu	Gly	
				325						330					335		
75	AAA	TAT	AGT	ATA	GAT	GTA	GAA	ACT	TTT	GAT	AAA	TTA	TAT	AAA	AGC	TTA	1056
	Lys	Tyr	Ser	Ile	Asp	Val	Glu	Ser	Phe	Asp	Lys	Leu	Tyr	Lys	Ser	Leu	
				340					345					350			
80	ATG	TTT	GGT	TTT	ACA	GAA	ACT	AAT	ATA	GCA	GAA	AAT	TAT	AAA	ATA	AAA	1104
	Met	Phe	Gly	Phe	Thr	Glu	Thr	Asn	Ile	Ala	Glu	Asn	Tyr	Lys	Ile	Lys	
			355					360					365				
85	ACT	AGA	GCT	TCT	TAT	TTT	AGT	GAT	TCC	TTA	CCA	CCA	GTA	AAA	ATA	AAA	1152
	Thr	Arg	Ala	Ser	Tyr	Phe	Ser	Asp	Ser	Leu	Pro	Pro	Val	Lys	Ile	Lys	
			370				375					380					

	AAT TTA TTA GAT AAT GAA ATC TAT ACT ATA GAG GAA GGG TTT AAT ATA Asn Leu Leu Asp Asn Glu Ile Tyr Thr Ile Glu Glu Gly Phe Asn Ile 385 390 395 400	1200
5	TCT GAT AAA GAT ATG GAA AAA GAA TAT AGA GGT CAG AAT AAA GCT ATA Ser Asp Lys Asp Met Glu Lys Glu Tyr Arg Gly Gln Asn Lys Ala Ile 405 410 415	1248
10	AAT AAA CAA GCT TAT GAA GAA ATT AGC AAG GAG CAT TTG GCT GTA TAT Asn Lys Gln Ala Tyr Glu Glu Ile Ser Lys Glu His Leu Ala Val Tyr 420 425 430	1296
15	AAG ATA CAA ATG TGT AAA AGT GTT AAA GCT CCA GGA ATA TGT ATT GAT Lys Ile Gln Met Cys Lys Ser Val Lys Ala Pro Gly Ile Cys Ile Asp 435 440 445	1344
20	GTT GAT AAT GAA GAT TTG TTC TTT ATA GCT GAT AAA AAT AGT TTT TCA Val Asp Asn Glu Asp Leu Phe Phe Ile Ala Asp Lys Asn Ser Phe Ser 450 455 460	1392
	GAT GAT TTA TCT AAA AAC GAA AGA ATA GAA TAT AAT ACA CAG AGT AAT Asp Asp Leu Ser Lys Asn Glu Arg Ile Glu Tyr Asn Thr Gln Ser Asn 465 470 475 480	1440
25	TAT ATA GAA AAT GAC TTC CCT ATA AAT GAA TTA ATT TTA GAT ACT GAT Tyr Ile Glu Asn Asp Phe Pro Ile Asn Glu Leu Ile Leu Asp Thr Asp 485 490 495	1488
30	TTA ATA AGT AAA ATA GAA TTA CCA AGT GAA AAT ACA GAA TCA CTT ACT Leu Ile Ser Lys Ile Glu Leu Pro Ser Glu Asn Thr Glu Ser Leu Thr 500 505 510	1536
35	GAT TTT AAT GTA GAT GTT CCA GTA TAT GAA AAA CAA CCC GCT ATA AAA Asp Phe Asn Val Asp Val Pro Val Tyr Glu Lys Gln Pro Ala Ile Lys 515 520 525	1584
40	AAA ATT TTT ACA GAT GAA AAT ACC ATC TTT CAA TAT TTA TAC TCT CAG Lys Ile Phe Thr Asp Glu Asn Thr Ile Phe Gln Tyr Leu Tyr Ser Gln 530 535 540	1632
	ACA TTT CTC TTA GAT ATA AGA GAT ATA AGT TTA ACA TCT TCA TTT GAT Thr Phe Leu Leu Asp Ile Arg Asp Ile Ser Leu Thr Ser Ser Phe Asp 545 550 555 560	1680
45	GAT GCA TTA TTA TTT TCT AAC AAA GTT TAT TCA TTT TTT TCT ATG GAT Asp Ala Leu Leu Phe Ser Asn Lys Val Tyr Ser Phe Phe Ser Met Asp 565 570 575	1728
50	TAT ATT AAA ACT GCT AAT AAA GTG GTA GAA GCA GGA TTA TTT GCA GGT Tyr Ile Lys Thr Ala Asn Lys Val Val Glu Ala Gly Leu Phe Ala Gly 580 585 590	1776
55	TGG GTG AAA CAG ATA GTA AAT GAT TTT GTA ATC GAA GCT AAT AAA AGC Trp Val Lys Gln Ile Val Asn Asp Phe Val Ile Glu Ala Asn Lys Ser 595 600 605	1824
60	AAT ACT ATG GAT AAA ATT GCA GAT ATA TCT CTA ATT GTT CCT TAT ATA Asn Thr Met Asp Lys Ile Ala Asp Ile Ser Leu Ile Val Pro Tyr Ile 610 615 620	1872
	GGA TTA GCT TTA AAT GTA GGA AAT GAA ACA GCT AAA GGA AAT TTT GAA Gly Leu Ala Leu Asn Val Gly Asn Glu Thr Ala Lys Gly Asn Phe Glu 625 630 635 640	1920
65	AAT GCT TTT GAG ATT GCA GGA GCC AGT ATT CTA CTA GAA TTT ATA CCA Asn Ala Phe Glu Ile Ala Gly Ala Ser Ile Leu Leu Glu Phe Ile Pro 645 650 655	1968
70	GAA CTT TTA ATA CCT GTA GTT GGA GCC TTT TTA TTA GAA TCA TAT ATT Glu Leu Leu Ile Pro Val Val Gly Ala Phe Leu Leu Glu Ser Tyr Ile	2016

	660	665	670	
5	GAC AAT AAA AAT AAA ATT ATT AAA Asp Asn Lys Asn Lys Ile Ile Lys 675 680	ACA ATA GAT AAT GCT TTA ACT AAA Thr Ile Asp Asn Ala Leu Thr Lys 685	2064	
10	AGA AAT GAA AAA TGG AGT GAT ATG TAC GGA TTA ATA GTA GCG CAA TGG Arg Asn Glu Lys Trp Ser Asp Met Tyr Gly Leu Ile Val Ala Gln Trp 690 695 700		2112	
15	CTC TCA ACA GTT AAT ACT CAA TTT TAT ACA ATA AAA GAG GGA ATG TAT Leu Ser Thr Val Asn Thr Gln Phe Tyr Thr Ile Lys Glu Gly Met Tyr 705 710 715 720		2160	
20	AAG GCT TTA AAT TAT CAA GCA CAA GCA TTG GAA GAA ATA ATA AAA TAC Lys Ala Leu Asn Tyr Gln Ala Gln Ala Leu Glu Glu Ile Ile Lys Tyr 725 730 735		2208	
25	AGA TAT AAT ATA TAT TCT GAA AAA GAA AAG TCA AAT ATT AAC ATC GAT Arg Tyr Asn Ile Tyr Ser Glu Lys Glu Lys Ser Asn Ile Asn Ile Asp 740 745 750		2256	
30	TTT AAT GAT ATA AAT TCT AAA CTT AAT GAG GGT ATT AAC CAA GCT ATA Phe Asn Asp Ile Asn Ser Lys Leu Asn Glu Gly Ile Asn Gln Ala Ile 755 760 765		2304	
35	GAT AAT ATA AAT AAT TTT ATA AAT GGA TGT TCT GTA TCA TAT TTA ATG Asp Asn Ile Asn Asn Phe Ile Asn Gly Cys Ser Val Ser Tyr Leu Met 770 775 780		2352	
40	AAA AAA ATG ATT CCA TTA GCT GTA GAA AAA TTA CTA GAC TTT GAT AAT Lys Lys Met Ile Pro Leu Ala Val Glu Lys Leu Leu Asp Phe Asp Asn 785 790 795 800		2400	
45	ACT CTC AAA AAA AAT TTG TTA AAT TAT ATA GAT GAA AAT AAA TTA TAT Thr Leu Lys Lys Asn Leu Leu Asn Tyr Ile Asp Glu Asn Lys Leu Tyr 805 810 815		2448	
50	TTG ATT GGA AGT GCA GAA TAT GAA AAA TCA AAA GTA AAT AAA TAC TTG Leu Ile Gly Ser Ala Glu Tyr Glu Lys Ser Lys Val Asn Lys Tyr Leu 820 825 830		2496	
55	AAA ACC ATT ATG CCG TTT GAT CTT TCA ATA TAT ACC AAT GAT ACA ATA Lys Thr Ile Met Pro Phe Asp Leu Ser Ile Tyr Thr Asn Asp Thr Ile 835 840 845		2544	
60	CTA ATA GAA ATG TTT AAT AAA TAT AAT AGC GAA ATT TTA AAT AAT ATT Leu Ile Glu Met Phe Asn Lys Tyr Asn Ser Glu Ile Leu Asn Asn Ile 850 855 860		2592	
65	ATC TTA AAT TTA AGA TAT AAG GAT AAT AAT TTA ATA GAT TTA TCA GGA Ile Leu Asn Leu Arg Tyr Lys Asp Asn Asn Leu Ile Asp Leu Ser Gly 865 870 875 880		2640	
70	TAT GGG GCA AAG GTA GAG GTA TAT GAT GGA GTC GAG CTT AAT GAT AAA Tyr Gly Ala Lys Val Glu Val Tyr Asp Gly Val Glu Leu Asn Asp Lys 885 890 895		2688	
75	AAT CAA TTT AAA TTA ACT AGT TCA GCA AAT AGT AAG ATT AGA GTG ACT Asn Gln Phe Lys Leu Thr Ser Ser Ala Asn Ser Lys Ile Arg Val Thr 900 905 910		2736	
80	CAA AAT CAG AAT ATC ATA TTT AAT AGT GTG TTC CTT GAT TTT AGC GTT Gln Asn Gln Asn Ile Ile Phe Asn Ser Val Phe Leu Asp Phe Ser Val 915 920 925		2784	
85	AGC TTT TGG ATA AGA ATA CCT AAA TAT AAG AAT GAT GGT ATA CAA AAT Ser Phe Trp Ile Arg Ile Pro Lys Tyr Lys Asn Asp Gly Ile Gln Asn 930 935 940		2832	

	TAT ATT CAT AAT GAA TAT ACA ATA ATT AAT TGT ATG AAA AAT AAT TCG Tyr Ile His Asn Glu Tyr Thr Ile Ile Asn Cys Met Lys Asn Asn Ser 945 950 955 960	2880
5	GGC TGG AAA ATA TCT ATT AGG GGT AAT AGG ATA ATA TGG ACT TTA ATT Gly Trp Lys Ile Ser Ile Arg Gly Asn Arg Ile Ile Trp Thr Leu Ile 965 970 975	2928
10	GAT ATA AAT GGA AAA ACC AAA TCG GTA TTT TTT GAA TAT AAC ATA AGA Asp Ile Asn Gly Lys Thr Lys Ser Val Phe Phe Glu Tyr Asn Ile Arg 980 985 990	2976
15	GAA GAT ATA TCA GAG TAT ATA AAT AGA TGG TTT TTT GTA ACT ATT ACT Glu Asp Ile Ser Glu Tyr Ile Asn Arg Trp Phe Phe Val Thr Ile Thr 995 1000 1005	3024
20	AAT AAT TTG AAT AAC GCT AAA ATT TAT ATT AAT GGT AAG CTA GAA TCA Asn Asn Leu Asn Asn Ala Lys Ile Tyr Ile Asn Gly Lys Leu Glu Ser 1010 1015 1020	3072
	AAT ACA GAT ATT AAA GAT ATA AGA GAA GTT ATT GCT AAT GGT GAA ATA Asn Thr Asp Ile Lys Asp Ile Arg Glu Val Ile Ala Asn Gly Glu Ile 1025 1030 1035 1040	3120
25	ATA TTT AAA TTA GAT GGT GAT ATA GAT AGA ACA CAA TTT ATT TGG ATG Ile Phe Lys Leu Asp Gly Asp Ile Asp Arg Thr Gln Phe Ile Trp Met 1045 1050 1055	3168
30	AAA TAT TTC AGT ATT TTT AAT ACG GAA TTA AGT CAA TCA AAT ATT GAA Lys Tyr Phe Ser Ile Phe Asn Thr Glu Leu Ser Gln Ser Asn Ile Glu 1060 1065 1070	3216
35	GAA AGA TAT AAA ATT CAA TCA TAT AGC GAA TAT TTA AAA GAT TTT TGG Glu Arg Tyr Lys Ile Gln Ser Tyr Ser Glu Tyr Leu Lys Asp Phe Trp 1075 1080 1085	3264
40	GGA AAT CCT TTA ATG TAC AAT AAA GAA TAT TAT ATG TTT AAT GCG GGG Gly Asn Pro Leu Met Tyr Asn Lys Glu Tyr Tyr Met Phe Asn Ala Gly 1090 1095 1100	3312
	AAT AAA AAT TCA TAT ATT AAA CTA AAG AAA GAT TCA CCT GTA GGT GAA Asn Lys Asn Ser Tyr Ile Lys Leu Lys Lys Asp Ser Pro Val Gly Glu 1105 1110 1115 1120	3360
45	ATT TTA ACA CGT AGC AAA TAT AAT CAA AAT TCT AAA TAT ATA AAT TAT Ile Leu Thr Arg Ser Lys Tyr Asn Gln Asn Ser Lys Tyr Ile Asn Tyr 1125 1130 1135	3408
50	AGA GAT TTA TAT ATT GGA GAA AAA TTT ATT ATA AGA AGA AAG TCA AAT Arg Asp Leu Tyr Ile Gly Glu Lys Phe Ile Arg Arg Lys Ser Asn 1140 1145 1150	3456
55	TCT CAA TCT ATA AAT GAT GAT ATA GTT AGA AAA GAA GAT TAT ATA TAT Ser Gln Ser Ile Asn Asp Asp Ile Val Arg Lys Glu Asp Tyr Ile Tyr 1155 1160 1165	3504
60	CTA GAT TTT TTT AAT TTA AAT CAA GAG TGG AGA GTA TAT ACC TAT AAA Leu Asp Phe Phe Asn Leu Asn Gln Glu Trp Arg Val Tyr Thr Tyr Lys 1170 1175 1180	3552
	TAT TTT AAG AAA GAG GAA GAA AAA TTG TTT TTA GCT CCT ATA AGT GAT Tyr Phe Lys Lys Glu Glu Glu Lys Leu Phe Leu Ala Pro Ile Ser Asp 1185 1190 1195 1200	3600
65	TCT GAT GAG TTT TAC AAT ACT ATA CAA ATA AAA GAA TAT GAT GAA CAG Ser Asp Glu Phe Tyr Asn Thr Ile Gln Ile Lys Glu Tyr Asp Glu Gln 1205 1210 1215	3648

	CCA ACA TAT AGT TGT CAG TTG CTT TTT AAA AAA GAT GAA GAA AGT ACT	3696
	Pro Thr Tyr Ser Cys Gln Leu Leu Phe Lys Lys Asp Glu Glu Ser Thr	
	1220 1225 1230	
5	GAT GAG ATA GGA TTG ATT GGT ATT CAT CGT TTC TAC GAA TCT GGA ATT	3744
	Asp Glu Ile Gly Leu Ile Gly Ile His Arg Phe Tyr Glu Ser Gly Ile	
	1235 1240 1245	
10	GTA TTT GAA GAG TAT AAA GAT TAT TTT TGT ATA AGT AAA TGG TAC TTA	3792
	Val Phe Glu Glu Tyr Lys Asp Tyr Phe Cys Ile Ser Lys Trp Tyr Leu	
	1250 1255 1260	
15	AAA GAG GTA AAA AGG AAA CCA TAT AAT TTA AAA TTG GGA TGT AAT TGG	3840
	Lys Glu Val Lys Arg Lys Pro Tyr Asn Leu Lys Leu Gly Cys Asn Trp	
	1265 1270 1275 1280	
20	CAG TTT ATT CCT AAA GAT GAA GGG TGG ACT GAA TAA	3876
	Gln Phe Ile Pro Lys Asp Glu Gly Trp Thr Glu	
	1285 1290	
	(2) INFORMATION FOR SEQ ID NO:42:	
	(i) SEQUENCE CHARACTERISTICS:	
25	(A) LENGTH: 1291 amino acids	
	(B) TYPE: amino acid	
	(D) TOPOLOGY: linear	
	(ii) MOLECULE TYPE: protein	
30	(x1) SEQUENCE DESCRIPTION: SEQ ID NO:42:	
	Met Pro Val Thr Ile Asn Asn Phe Asn Tyr Asn Asp Pro Ile Asp Asn	
	1 5 10 15	
35	Asn Asn Ile Ile Met Met Glu Pro Pro Phe Ala Arg Gly Thr Gly Arg	
	20 25 30	
40	Tyr Tyr Lys Ala Phe Lys Ile Thr Asp Arg Ile Trp Ile Ile Pro Glu	
	35 40 45	
	Arg Tyr Thr Phe Gly Tyr Lys Pro Glu Asp Phe Asn Lys Ser Ser Gly	
	50 55 60	
45	Ile Phe Asn Arg Asp Val Cys Glu Tyr Tyr Asp Pro Asp Tyr Leu Asn	
	65 70 75 80	
	Thr Asn Asp Lys Lys Asn Ile Phe Leu Gln Thr Met Ile Lys Leu Phe	
	85 90 95	
50	Asn Arg Ile Lys Ser Lys Pro Leu Gly Glu Lys Leu Leu Glu Met Ile	
	100 105 110	
55	Ile Asn Gly Ile Pro Tyr Leu Gly Asp Arg Arg Val Pro Leu Glu Glu	
	115 120 125	
	Phe Asn Thr Asn Ile Ala Ser Val Thr Val Asn Lys Leu Ile Ser Asn	
	130 135 140	
60	Pro Gly Glu Val Glu Arg Lys Lys Gly Ile Phe Ala Asn Leu Ile Ile	
	145 150 155 160	

Phe Gly Pro Gly Pro Val Leu Asn Glu Asn Glu Thr Ile Asp Ile Gly  
 165 170 175  
 5 Ile Gln Asn His Phe Ala Ser Arg Glu Gly Phe Gly Gly Ile Met Gln  
 180 185 190  
 Met Lys Phe Cys Pro Glu Tyr Val Ser Val Phe Asn Asn Val Gln Glu  
 195 200 205  
 10 Asn Lys Gly Ala Ser Ile Phe Asn Arg Arg Gly Tyr Phe Ser Asp Pro  
 210 215 220  
 Ala Leu Ile Leu Met His Glu Leu Ile His Val Leu His Gly Leu Tyr  
 225 230 235 240  
 15 Gly Ile Lys Val Asp Asp Leu Pro Ile Val Pro Asn Glu Lys Lys Phe  
 245 250 255  
 20 Phe Met Gln Ser Thr Asp Ala Ile Gln Ala Glu Glu Leu Tyr Thr Phe  
 260 265 270  
 Gly Gly Gln Asp Pro Ser Ile Ile Thr Pro Ser Thr Asp Lys Ser Ile  
 275 280 285  
 25 Tyr Asp Lys Val Leu Gln Asn Phe Arg Gly Ile Val Asp Arg Leu Asn  
 290 295 300  
 Lys Val Leu Val Cys Ile Ser Asp Pro Asn Ile Asn Ile Asn Ile Tyr  
 305 310 315 320  
 30 Lys Asn Lys Phe Lys Asp Lys Tyr Lys Phe Val Glu Asp Ser Glu Gly  
 325 330 335  
 35 Lys Tyr Ser Ile Asp Val Glu Ser Phe Asp Lys Leu Tyr Lys Ser Leu  
 340 345 350  
 Met Phe Gly Phe Thr Glu Thr Asn Ile Ala Glu Asn Tyr Lys Ile Lys  
 355 360 365  
 40 Thr Arg Ala Ser Tyr Phe Ser Asp Ser Leu Pro Pro Val Lys Ile Lys  
 370 375 380  
 Asn Leu Leu Asp Asn Glu Ile Tyr Thr Ile Glu Glu Gly Phe Asn Ile  
 385 390 395 400  
 45 Ser Asp Lys Asp Met Glu Lys Glu Tyr Arg Gly Gln Asn Lys Ala Ile  
 405 410 415  
 50 Asn Lys Gln Ala Tyr Glu Glu Ile Ser Lys Glu His Leu Ala Val Tyr  
 420 425 430  
 Lys Ile Gln Met Cys Lys Ser Val Lys Ala Pro Gly Ile Cys Ile Asp  
 435 440 445  
 55 Val Asp Asn Glu Asp Leu Phe Phe Ile Ala Asp Lys Asn Ser Phe Ser  
 450 455 460  
 Asp Asp Leu Ser Lys Asn Glu Arg Ile Glu Tyr Asn Thr Gln Ser Asn  
 465 470 475 480  
 60 Tyr Ile Glu Asn Asp Phe Pro Ile Asn Glu Leu Ile Leu Asp Thr Asp  
 485 490 495  
 65 Leu Ile Ser Lys Ile Glu Leu Pro Ser Glu Asn Thr Glu Ser Leu Thr  
 500 505 510  
 Asp Phe Asn Val Asp Val Pro Val Tyr Glu Lys Gln Pro Ala Ile Lys  
 515 520 525  
 70 Lys Ile Phe Thr Asp Glu Asn Thr Ile Phe Gln Tyr Leu Tyr Ser Gln

	530	535	540
5	Thr Phe Leu Leu Asp 545	Ile Arg Asp Ile Ser 550	Leu Thr Ser Ser Phe Asp 555 560
	Asp Ala Leu Leu Phe 565	Ser Asn Lys Val Tyr 570	Ser Phe Phe Ser Met Asp 575
10	Tyr Ile Lys Thr 580	Ala Asn Lys Val Val 585	Glu Ala Gly Leu Phe Ala Gly 590
	Trp Val Lys Gln 595	Ile Val Asn Asp Phe 600	Val Ile Glu Ala Asn Lys Ser 605
15	Asn Thr Met Asp Lys 610	Ile Ala Asp Ile Ser 615	Leu Ile Val Pro Tyr Ile 620
	Gly Leu Ala Leu Asn 625	Val Gly Asn Glu Thr 630	Ala Lys Gly Asn Phe Glu 635 640
20	Asn Ala Phe Glu 645	Ile Ala Gly Ala Ser 650	Ile Leu Leu Glu Phe Ile Pro 655
25	Glu Leu Leu Ile 660	Pro Val Val Gly 665	Ala Phe Leu Leu Glu Ser Tyr Ile 670
	Asp Asn Lys Asn 675	Lys Ile Ile Lys Thr 680	Ile Asp Asn Ala Leu Thr Lys 685
30	Arg Asn Glu Lys Trp 690	Ser Asp Met Tyr 695	Gly Leu Ile Val Ala Gln Trp 700
	Leu Ser Thr Val 705	Asn Thr Gln Phe Tyr 710	Thr Ile Lys Glu Gly Met Tyr 715 720
35	Lys Ala Leu Asn Tyr 725	Gln Ala Gln Ala 730	Leu Glu Glu Ile Ile Lys Tyr 735
40	Arg Tyr Asn Ile 740	Tyr Ser Glu Lys 745	Glu Lys Ser Asn Ile Asn Ile Asp 750
	Phe Asn Asp Ile 755	Asn Ser Lys Leu 760	Asn Glu Gly Ile Asn Gln Ala Ile 765
45	Asp Asn Ile Asn 770	Asn Phe Ile Asn 775	Gly Cys Ser Val Ser Tyr Leu Met 780
	Lys Lys Met Ile 785	Pro Leu Ala Val 790	Glu Lys Leu Leu Asp Phe Asp Asn 795 800
50	Thr Leu Lys Lys 805	Asn Leu Leu Asn 810	Tyr Ile Asp Glu Asn Lys Leu Tyr 815
55	Leu Ile Gly Ser 820	Ala Glu Tyr Glu 825	Lys Ser Lys Val Asn Lys Tyr Leu 830
	Lys Thr Ile Met 835	Pro Phe Asp Leu 840	Ser Ile Tyr Thr Asn Asp Thr Ile 845
60	Leu Ile Glu Met 850	Phe Asn Lys Tyr 855	Asn Ser Glu Ile Leu Asn Asn Ile 860
	Ile Leu Asn Leu 865	Arg Tyr Lys Asp 870	Asn Asn Leu Ile Asp Leu Ser Gly 875 880
65	Tyr Gly Ala Lys 885	Val Glu Val Tyr 890	Asp Gly Val Glu Leu Asn Asp Lys 895
70	Asn Gln Phe Lys 900	Leu Thr Ser Ser 905	Ala Asn Ser Lys Ile Arg Val Thr 910



Gln Asn Gln Asn Ile Ile Phe Asn Ser Val Phe Leu Asp Phe Ser Val  
 915 920 925  
 5 Ser Phe Trp Ile Arg Ile Pro Lys Tyr Lys Asn Asp Gly Ile Gln Asn  
 930 935 940  
 Tyr Ile His Asn Glu Tyr Thr Ile Ile Asn Cys Met Lys Asn Asn Ser  
 945 950 955 960  
 10 Gly Trp Lys Ile Ser Ile Arg Gly Asn Arg Ile Ile Trp Thr Leu Ile  
 965 970 975  
 Asp Ile Asn Gly Lys Thr Lys Ser Val Phe Phe Glu Tyr Asn Ile Arg  
 980 985 990  
 15 Glu Asp Ile Ser Glu Tyr Ile Asn Arg Trp Phe Phe Val Thr Ile Thr  
 995 1000 1005  
 Asn Asn Leu Asn Asn Ala Lys Ile Tyr Ile Asn Gly Lys Leu Glu Ser  
 1010 1015 1020  
 Asn Thr Asp Ile Lys Asp Ile Arg Glu Val Ile Ala Asn Gly Glu Ile  
 1025 1030 1035 1040  
 25 Ile Phe Lys Leu Asp Gly Asp Ile Asp Arg Thr Gln Phe Ile Trp Met  
 1045 1050 1055  
 Lys Tyr Phe Ser Ile Phe Asn Thr Glu Leu Ser Gln Ser Asn Ile Glu  
 1060 1065 1070  
 30 Glu Arg Tyr Lys Ile Gln Ser Tyr Ser Glu Tyr Leu Lys Asp Phe Trp  
 1075 1080 1085  
 Gly Asn Pro Leu Met Tyr Asn Lys Glu Tyr Tyr Met Phe Asn Ala Gly  
 1090 1095 1100  
 Asn Lys Asn Ser Tyr Ile Lys Leu Lys Lys Asp Ser Pro Val Gly Glu  
 1105 1110 1115 1120  
 40 Ile Leu Thr Arg Ser Lys Tyr Asn Gln Asn Ser Lys Tyr Ile Asn Tyr  
 1125 1130 1135  
 Arg Asp Leu Tyr Ile Gly Glu Lys Phe Ile Ile Arg Arg Lys Ser Asn  
 1140 1145 1150  
 45 Ser Gln Ser Ile Asn Asp Asp Ile Val Arg Lys Glu Asp Tyr Ile Tyr  
 1155 1160 1165  
 Leu Asp Phe Phe Asn Leu Asn Gln Glu Trp Arg Val Tyr Thr Tyr Lys  
 1170 1175 1180  
 Tyr Phe Lys Lys Glu Glu Glu Lys Leu Phe Leu Ala Pro Ile Ser Asp  
 1185 1190 1195 1200  
 55 Ser Asp Glu Phe Tyr Asn Thr Ile Gln Ile Lys Glu Tyr Asp Glu Gln  
 1205 1210 1215  
 Pro Thr Tyr Ser Cys Gln Leu Leu Phe Lys Lys Asp Glu Glu Ser Thr  
 1220 1225 1230  
 60 Asp Glu Ile Gly Leu Ile Gly Ile His Arg Phe Tyr Glu Ser Gly Ile  
 1235 1240 1245  
 Val Phe Glu Glu Tyr Lys Asp Tyr Phe Cys Ile Ser Lys Trp Tyr Leu  
 1250 1255 1260  
 Lys Glu Val Lys Arg Lys Pro Tyr Asn Leu Lys Leu Gly Cys Asn Trp  
 1265 1270 1275 1280  
 70 Gln Phe Ile Pro Lys Asp Glu Gly Trp Thr Glu

1285

1290

## (2) INFORMATION FOR SEQ ID NO:43:

5 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 1526 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: other nucleic acid  
 (A) DESCRIPTION: /desc = "DNA"

15 (ix) FEATURE:  
 (A) NAME/KEY: CDS  
 (B) LOCATION: 108..1523

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

20	AGATCTCGAT CCCGCGAAAT TAATACGACT CACTATAGGG GAATTGTGAG CGGATAACAA	60
	TTCCCCCTCTA GAAATAATTT TGTTTAACTT TAAGAAGGAG ATATAACC ATG GGC CAT	116
		Met Gly His
25		1
	CAT CAT CAT CAT CAT CAT CAT CAT CAC AGC AGC GGC CAT ATC GAA GGT	164
	His His His His His His His His His Ser Ser Gly His Ile Glu Gly	
	5 10 15	
30	CGT CAT ATG GCT AGC ATG GCT GAT ACA ATA CTA ATA GAA ATG TTT AAT	212
	Arg His Met Ala Ser Met Ala Asp Thr Ile Leu Ile Glu Met Phe Asn	35
	20 25 30	
35	AAA TAT AAT AGC GAA ATT TTA AAT AAT ATT ATC TTA AAT TTA AGA TAT	260
	Lys Tyr Asn Ser Glu Ile Leu Asn Asn Ile Ile Leu Asn Leu Arg Tyr	50
	40 45 50	
40	AGA GAT AAT AAT TTA ATA GAT TTA TCA GGA TAT GGA GCA AAG GTA GAG	308
	Arg Asp Asn Asn Leu Ile Asp Leu Ser Gly Tyr Gly Ala Lys Val Glu	65
	55 60	
45	GTA TAT GAT GGG GTC AAG CTT AAT GAT AAA AAT CAA TTT AAA TTA ACT	356
	Val Tyr Asp Gly Val Lys Leu Asn Asp Lys Asn Gln Phe Lys Leu Thr	80
	70 75 80	
50	AGT TCA GCA GAT AGT AAG ATT AGA GTC ACT CAA AAT CAG AAT ATT ATA	404
	Ser Ser Ala Asp Ser Lys Ile Arg Val Thr Gln Asn Gln Asn Ile Ile	95
	85 90 95	
55	TTT AAT AGT ATG TTC CTT GAT TTT AGC GTT AGC TTT TGG ATA AGG ATA	452
	Phe Asn Ser Met Phe Leu Asp Phe Ser Val Ser Phe Trp Ile Arg Ile	115
	100 105 110 115	
60	CCT AAA TAT AGG AAT GAT GAT ATA CAA AAT TAT ATT CAT AAT GAA TAT	500
	Pro Lys Tyr Arg Asn Asp Asp Ile Gln Asn Tyr Ile His Asn Glu Tyr	130
	120 125 130	
65	ACG ATA ATT AAT TGT ATG AAA AAT AAT TCA GGC TGG AAA ATA TCT ATT	548
	Thr Ile Ile Asn Cys Met Lys Asn Asn Ser Gly Trp Lys Ile Ser Ile	145
	135 140 145	
70	AGG GCT AAT AGG ATA ATA TGG ACC TTA ATT GAT ATA AAT GGA AAA ACC	596
	Arg Gly Asn Arg Ile Ile Trp Thr Leu Ile Asp Ile Asn Gly Lys Thr	160
	150 155 160	
75	AAA TCA GTA TTT TTT GAA TAT AAC ATA AGA GAA GAT ATA TCA GAG TAT	644
	Lys Ser Val Phe Phe Glu Tyr Asn Ile Arg Glu Asp Ile Ser Glu Tyr	175
	165 170 175	
80	ATA AAT AGA TGG TTT TTT GTA ACT ATT ACT AAT AAT TTG GAT AAT GCT	692

	Ile Asn Arg Trp Phe Phe Val Thr Ile Thr Asn Asn Leu Asp Asn Ala	
	180 185 190 195	
5	AAA ATT TAT ATT AAT GGC ACG TTA GAA TCA AAT ATG GAT ATT AAA GAT Lys Ile Tyr Ile Asn Gly Thr Leu Glu Ser Asn Met Asp Ile Lys Asp	740
	200 205 210	
10	ATA GGA GAA GTT ATT GTT AAT GGT GAA ATA ACA TTT AAA TTA GAT GGT Ile Gly Glu Val Ile Val Asn Gly Glu Ile Thr Phe Lys Leu Asp Gly	788
	215 220 225	
15	GAT GTA GAT AGA ACA CAA TTT ATT TGG ATG AAA TAT TTT AGT ATT TTT Asp Val Asp Arg Thr Gln Phe Ile Trp Met Lys Tyr Phe Ser Ile Phe	836
	230 235 240	
	AAT ACG CAA TTA AAT CAA TCA AAT ATT AAA GAG ATA TAT AAA ATT CAA Asn Thr Gln Leu Asn Gln Ser Asn Ile Lys Glu Ile Tyr Lys Ile Gln	884
	245 250 255	
20	TCA TAT AGC GAA TAC TTA AAA GAT TTT TGG GGA AAT CCT TTA ATG TAT Ser Tyr Ser Glu Tyr Leu Lys Asp Phe Trp Gly Asn Pro Leu Met Tyr	932
	260 265 270 275	
25	AAT AAA GAA TAT TAT ATG TTT AAT GCG GGG AAT AAA AAT TCA TAT ATT Asn Lys Glu Tyr Tyr Met Phe Asn Ala Gly Asn Lys Asn Ser Tyr Ile	980
	280 285 290	
30	AAA CTA GTG AAA GAT TCA TCT GTA GGT GAA ATA TTA ATA CGT AGC AAA Lys Leu Val Lys Asp Ser Ser Val Gly Glu Ile Leu Ile Arg Ser Lys	1028
	295 300 305	
35	TAT AAT CAG AAT TCC AAT TAT ATA AAT TAT AGA AAT TTA TAT ATT GGA Tyr Asn Gln Asn Ser Asn Tyr Ile Asn Tyr Arg Asn Leu Tyr Ile Gly	1076
	310 315 320	
	GAA AAA TTT ATT ATA AGA AGA GAG TCA AAT TCT CAA TCT ATA AAT GAT Glu Lys Phe Ile Ile Arg Arg Glu Ser Asn Ser Gln Ser Ile Asn Asp	1124
	325 330 335	
40	GAT ATA GTT AGA AAA GAA GAT TAT ATA CAT CTA GAT TTG GTA CTT CAC Asp Ile Val Arg Lys Glu Asp Tyr Ile His Leu Asp Leu Val Leu His	1172
	340 345 350 355	
45	CAT GAA GAG TGG AGA GTA TAT GCC TAT AAA TAT TTT AAG GAA CAG GAA His Glu Glu Trp Arg Val Tyr Ala Tyr Lys Tyr Phe Lys Glu Gln Glu	1220
	360 365 370	
50	GAA AAA TTG TTT TTA TCT ATT ATA AGT GAT TCT AAT GAA TTT TAT AAG Glu Lys Leu Phe Leu Ser Ile Ile Ser Asp Ser Asn Glu Phe Tyr Lys	1268
	375 380 385	
55	ACT ATA GAA ATA AAA GAA TAT GAT GAA CAG CCA TCA TAT AGT TGT CAG Thr Ile Glu Ile Lys Glu Tyr Asp Glu Gln Pro Ser Tyr Ser Cys Gln	1316
	390 395 400	
	TTG CTT TTT AAA AAA GAT GAA GAA AGT ACT GAT GAT ATA GGA TTG ATT Leu Leu Phe Lys Lys Asp Glu Glu Ser Thr Asp Asp Ile Gly Leu Ile	1364
	405 410 415	
60	GGT ATT CAT CGT TTC TAC GAA TCT GGA GTT TTA CGT AAA AAG TAT AAA Gly Ile His Arg Phe Tyr Glu Ser Gly Val Leu Arg Lys Lys Tyr Lys	1412
	420 425 430 435	
65	GAT TAT TTT TGT ATA AGT AAA TGG TAC TTA AAA GAG GTA AAA AGG AAA Asp Tyr Phe Cys Ile Ser Lys Trp Tyr Leu Lys Glu Val Lys Arg Lys	1460
	440 445 450	
70	CCA TAT AAG TCA AAT TTG GGA TGT AAT TGG CAG TTT ATT CCT AAA GAT Pro Tyr Lys Ser Asn Leu Gly Cys Asn Trp Gln Phe Ile Pro Lys Asp	1508
	455 460 465	

GAA GGG TGG ACT GAA TAA  
 Glu Gly Trp Thr Glu  
 470

1526

5

(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 472 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

10

(ii) MOLECULE TYPE: protein

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

Met Gly His His His His His His His His His His Ser Ser Gly His  
 1 5 10 15  
 Ile Glu Gly Arg His Met Ala Ser Met Ala Asp Thr Ile Leu Ile Glu  
 20 25 30  
 Met Phe Asn Lys Tyr Asn Ser Glu Ile Leu Asn Asn Ile Ile Leu Asn  
 35 40 45  
 Leu Arg Tyr Arg Asp Asn Asn Leu Ile Asp Leu Ser Gly Tyr Gly Ala  
 50 55 60  
 Lys Val Glu Val Tyr Asp Gly Val Lys Leu Asn Asp Lys Asn Gln Phe  
 65 70 75 80  
 Lys Leu Thr Ser Ser Ala Asp Ser Lys Ile Arg Val Thr Gln Asn Gln  
 85 90 95  
 Asn Ile Ile Phe Asn Ser Met Phe Leu Asp Phe Ser Val Ser Phe Trp  
 100 105 110  
 Ile Arg Ile Pro Lys Tyr Arg Asn Asp Asp Ile Gln Asn Tyr Ile His  
 115 120 125  
 Asn Glu Tyr Thr Ile Ile Asn Cys Met Lys Asn Asn Ser Gly Trp Lys  
 130 135 140  
 Ile Ser Ile Arg Gly Asn Arg Ile Ile Trp Thr Leu Ile Asp Ile Asn  
 145 150 155 160  
 Gly Lys Thr Lys Ser Val Phe Phe Glu Tyr Asn Ile Arg Glu Asp Ile  
 165 170 175  
 Ser Glu Tyr Ile Asn Arg Trp Phe Phe Val Thr Ile Thr Asn Asn Leu  
 180 185 190  
 Asp Asn Ala Lys Ile Tyr Ile Asn Gly Thr Leu Glu Ser Asn Met Asp  
 195 200 205  
 Ile Lys Asp Ile Gly Glu Val Ile Val Asn Gly Glu Ile Thr Phe Lys  
 210 215 220  
 Leu Asp Gly Asp Val Asp Arg Thr Gln Phe Ile Trp Met Lys Tyr Phe  
 225 230 235 240  
 Ser Ile Phe Asn Thr Gln Leu Asn Gln Ser Asn Ile Lys Glu Ile Tyr  
 245 250 255  
 Lys Ile Gln Ser Tyr Ser Glu Tyr Leu Lys Asp Phe Trp Gly Asn Pro  
 260 265 270  
 Leu Met Tyr Asn Lys Glu Tyr Tyr Met Phe Asn Ala Gly Asn Lys Asn  
 275 280 285  
 Ser Tyr Ile Lys Leu Val Lys Asp Ser Ser Val Gly Glu Ile Leu Ile

	290	295	300	
	Arg Ser Lys Tyr Asn Gln Asn Ser Asn Tyr Ile Asn Tyr Arg Asn Leu			
5	305	310	315	320
	Tyr Ile Gly Glu Lys Phe Ile Ile Arg Arg Glu Ser Asn Ser Gln Ser			
	325	330	335	
10	Ile Asn Asp Asp Ile Val Arg Lys Glu Asp Tyr Ile His Leu Asp Leu			
	340	345	350	
	Val Leu His His Glu Glu Trp Arg Val Tyr Ala Tyr Lys Tyr Phe Lys			
	355	360	365	
15	Glu Gln Glu Glu Lys Leu Phe Leu Ser Ile Ile Ser Asp Ser Asn Glu			
	370	375	380	
	Phe Tyr Lys Thr Ile Glu Ile Lys Glu Tyr Asp Glu Gln Pro Ser Tyr			
20	385	390	395	400
	Ser Cys Gln Leu Leu Phe Lys Lys Asp Glu Glu Ser Thr Asp Asp Ile			
	405	410	415	
25	Gly Leu Ile Gly Ile His Arg Phe Tyr Glu Ser Gly Val Leu Arg Lys			
	420	425	430	
	Lys Tyr Lys Asp Tyr Phe Cys Ile Ser Lys Trp Tyr Leu Lys Glu Val			
	435	440	445	
30	Lys Arg Lys Pro Tyr Lys Ser Asn Leu Gly Cys Asn Trp Gln Phe Ile			
	450	455	460	
	Pro Lys Asp Glu Gly Trp Thr Glu			
35	465	470		
	(2) INFORMATION FOR SEQ ID NO:45:			
	(i) SEQUENCE CHARACTERISTICS:			
40	(A) LENGTH: 1547 base pairs			
	(B) TYPE: nucleic acid			
	(C) STRANDEDNESS: double			
	(D) TOPOLOGY: linear			
45	(ii) MOLECULE TYPE: DNA (genomic)			
	(ix) FEATURE:			
	(A) NAME/KEY: CDS			
	(B) LOCATION: 108..1523			
50	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:			
	AGATCTCGAT CCCGCGAAAT TAATACGACT CACTATAGGG GAATTGTGAG CGGATAACAA			60
55	TTCCCTCTA GAAATAATTT TGTTTAACTT TAAGAAGGAG ATATACC ATG GGC CAT			116
		Met Gly His		
		1		
60	CAT CAT CAT CAT CAT CAT CAT CAT CAC AGC AGC GGC CAT ATC GAA GGT			164
	His His His His His His His His His Ser Ser Gly His Ile Glu Gly			
	5	10	15	
	CGT CAT ATG GCT AGC ATG GCT GAT ACA ATA CTA ATA GAA ATG TTT AAT			212
65	Arg His Met Ala Ser Met Ala Asp Thr Ile Leu Ile Glu Met Phe Asn			
	20	25	30	35
	AAA TAT AAT AGC GAA ATT TTA AAT AAT ATT ATC TTA AAT TTA AGA TAT			260
	Lys Tyr Asn Ser Glu Ile Leu Asn Asn Ile Ile Leu Asn Leu Arg Tyr			
	40	45	50	
70	AAG GAT AAT AAT TTA ATA GAT TTA TCA GGA TAT GGG GCA AAG GTA GAG			308

	Lys	Asp	Asn	Asn	Leu	Ile	Asp	Leu	Ser	Gly	Tyr	Gly	Ala	Lys	Val	Glu	
				55					60					65			
5	GTA	TAT	GAT	GGA	GTC	GAG	CTT	AAT	GAT	AAA	AAT	CAA	TTT	AAA	TTA	ACT	356
	Val	Tyr	Asp	Gly	Val	Glu	Leu	Asn	Asp	Lys	Asn	Gln	Phe	Lys	Leu	Thr	
			70					75					80				
10	AGT	TCA	GCA	AAT	AGT	AAG	ATT	AGA	GTG	ACT	CAA	AAT	CAG	AAT	ATC	ATA	404
	Ser	Ser	Ala	Asn	Ser	Lys	Ile	Arg	Val	Thr	Gln	Asn	Gln	Asn	Ile	Ile	
			85					90				95					
15	TTT	AAT	AGT	GTG	TTC	CTT	GAT	TTT	AGC	GTT	AGC	TTT	TGG	ATA	AGA	ATA	452
	Phe	Asn	Ser	Val	Phe	Leu	Asp	Phe	Ser	Val	Ser	Phe	Trp	Ile	Arg	Ile	
	100					105					110					115	
	CCT	AAA	TAT	AAG	AAT	GAT	GGT	ATA	CAA	AAT	TAT	ATT	CAT	AAT	GAA	TAT	500
	Pro	Lys	Tyr	Lys	Asn	Asp	Gly	Ile	Gln	Asn	Tyr	Ile	His	Asn	Glu	Tyr	
					120					125					130		
20	ACA	ATA	ATT	AAT	TGT	ATG	AAA	AAT	AAT	TCG	GGC	TGG	AAA	ATA	TCT	ATT	548
	Thr	Ile	Ile	Asn	Cys	Met	Lys	Asn	Asn	Ser	Gly	Trp	Lys	Ile	Ser	Ile	
					135				140					145			
25	AGG	GGT	AAT	AGG	ATA	ATA	TGG	ACT	TTA	ATT	GAT	ATA	AAT	GGA	AAA	ACC	596
	Arg	Gly	Asn	Arg	Ile	Ile	Trp	Thr	Leu	Ile	Asp	Ile	Asn	Gly	Lys	Thr	
			150					155					160				
30	AAA	TCG	GTA	TTT	TTT	GAA	TAT	AAC	ATA	AGA	GAA	GAT	ATA	TCA	GAG	TAT	644
	Lys	Ser	Val	Phe	Phe	Glu	Tyr	Asn	Ile	Arg	Glu	Asp	Ile	Ser	Glu	Tyr	
			165				170					175					
35	ATA	AAT	AGA	TGG	TTT	TTT	GTA	ACT	ATT	ACT	AAT	AAT	TTG	AAT	AAC	GCT	692
	Ile	Asn	Arg	Trp	Phe	Phe	Val	Thr	Ile	Thr	Asn	Asn	Leu	Asn	Asn	Ala	
	180					185					190					195	
	AAA	ATT	TAT	ATT	AAT	GGT	AAG	CTA	GAA	TCA	AAT	ACA	GAT	ATT	AAA	GAT	740
	Lys	Ile	Tyr	Ile	Asn	Gly	Lys	Leu	Glu	Ser	Asn	Thr	Asp	Ile	Lys	Asp	
					200					205					210		
40	ATA	AGA	GAA	GTT	ATT	GCT	AAT	GGT	GAA	ATA	ATA	TTT	AAA	TTA	GAT	GGT	788
	Ile	Arg	Glu	Val	Ile	Ala	Asn	Gly	Glu	Ile	Ile	Phe	Lys	Leu	Asp	Gly	
				215					220					225			
45	GAT	ATA	GAT	AGA	ACA	CAA	TTT	ATT	TGG	ATG	AAA	TAT	TTC	AGT	ATT	TTT	836
	Asp	Ile	Asp	Arg	Thr	Gln	Phe	Ile	Trp	Met	Lys	Tyr	Phe	Ser	Ile	Phe	
				230				235					240				
50	AAT	ACG	GAA	TTA	AGT	CAA	TCA	AAT	ATT	GAA	GAA	AGA	TAT	AAA	ATT	CAA	884
	Asn	Thr	Glu	Leu	Ser	Gln	Ser	Asn	Ile	Glu	Glu	Arg	Tyr	Lys	Ile	Gln	
			245				250					255					
55	TCA	TAT	AGC	GAA	TAT	TTA	AAA	GAT	TTT	TGG	GGA	AAT	CCT	TTA	ATG	TAC	932
	Ser	Tyr	Ser	Glu	Tyr	Leu	Lys	Asp	Phe	Trp	Gly	Asn	Pro	Leu	Met	Tyr	
	260					265					270					275	
	AAT	AAA	GAA	TAT	TAT	ATG	TTT	AAT	GCG	GGG	AAT	AAA	AAT	TCA	TAT	ATT	980
	Asn	Lys	Glu	Tyr	Tyr	Met	Phe	Asn	Ala	Gly	Asn	Lys	Asn	Ser	Tyr	Ile	
					280					285					290		
60	AAA	CTA	AAG	AAA	GAT	TCA	CCT	GTA	GGT	GAA	ATT	TTA	ACA	CGT	AGC	AAA	1028
	Lys	Leu	Lys	Lys	Asp	Ser	Pro	Val	Gly	Glu	Ile	Leu	Thr	Arg	Ser	Lys	
				295					300					305			
65	TAT	AAT	CAA	AAT	TCT	AAA	TAT	ATA	AAT	TAT	AGA	GAT	TTA	TAT	ATT	GGA	1076
	Tyr	Asn	Gln	Asn	Ser	Lys	Tyr	Ile	Asn	Tyr	Arg	Asp	Leu	Tyr	Ile	Gly	
			310					315					320				

	GAA AAA TTT ATT ATA AGA AGA AAG TCA AAT TCT CAA TCT ATA AAT GAT Glu Lys Phe Ile Ile Arg Arg Lys Ser Asn Ser Gln Ser Ile Asn Asp 325 330 335	1124
5	GAT ATA GTT AGA AAA GAA GAT TAT ATA TAT CTA GAT TTT TTT AAT TTA Asp Ile Val Arg Lys Glu Asp Tyr Ile Tyr Leu Asp Phe Phe Asn Leu 340 345 350 355	1172
10	AAT CAA GAG TGG AGA GTA TAT ACC TAT AAA TAT TTT AAG AAA GAG GAA Asn Gln Glu Trp Arg Val Tyr Thr Tyr Lys Tyr Phe Lys Lys Glu Glu 360 365 370	1220
15	GAA AAA TTG TTT TTA GCT CCT ATA AGT GAT TCT GAT GAG TTT TAC AAT Glu Lys Leu Phe Leu Ala Pro Ile Ser Asp Ser Asp Glu Phe Tyr Asn 375 380 385	1268
20	ACT ATA CAA ATA AAA GAA TAT GAT GAA CAG CCA ACA TAT AGT TGT CAG Thr Ile Gln Ile Lys Glu Tyr Asp Glu Gln Pro Thr Tyr Ser Cys Gln 390 395 400	1316
	TTG CTT TTT AAA AAA GAT GAA GAA AGT ACT GAT GAG ATA GGA TTG ATT Leu Leu Phe Lys Lys Asp Glu Glu Ser Thr Asp Glu Ile Gly Leu Ile 405 410 415	1364
25	GGT ATT CAT CGT TTC TAC GAA TCT GGA ATT GTA TTT GAA GAG TAT AAA Gly Ile His Arg Phe Tyr Glu Ser Gly Ile Val Phe Glu Glu Tyr Lys 420 425 430 435	1412
30	GAT TAT TTT TGT ATA AGT AAA TGG TAC TTA AAA GAG GTA AAA AGG AAA Asp Tyr Phe Cys Ile Ser Lys Trp Tyr Leu Lys Glu Val Lys Arg Lys 440 445 450	1460
35	CCA TAT AAT TTA AAA TTG GGA TGT AAT TGG CAG TTT ATT CCT AAA GAT Pro Tyr Asn Leu Lys Leu Gly Cys Asn Trp Gln Phe Ile Pro Lys Asp 455 460 465	1508
40	GAA GGG TGG ACT GAA TAAAAGCTTG CGGCCGCACT CGAG Glu Gly Trp Thr Glu 470	1547
(2) INFORMATION FOR SEQ ID NO:46:		
(i) SEQUENCE CHARACTERISTICS:		
45	(A) LENGTH: 472 amino acids	
	(B) TYPE: amino acid	
	(D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: protein		
50	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:	
	Met Gly His His His His His His His His His Ser Ser Gly His 1 5 10 15	
55	Ile Glu Gly Arg His Met Ala Ser Met Ala Asp Thr Ile Leu Ile Glu 20 25 30	
	Met Phe Asn Lys Tyr Asn Ser Glu Ile Leu Asn Asn Ile Ile Leu Asn 35 40 45	
60	Leu Arg Tyr Lys Asp Asn Asn Leu Ile Asp Leu Ser Gly Tyr Gly Ala 50 55 60	
65	Lys Val Glu Val Tyr Asp Gly Val Glu Leu Asn Asp Lys Asn Gln Phe 65 70 75 80	
	Lys Leu Thr Ser Ser Ala Asn Ser Lys Ile Arg Val Thr Gln Asn Gln 85 90 95	
70	Asn Ile Ile Phe Asn Ser Val Phe Leu Asp Phe Ser Val Ser Phe Trp	

	100	105	110
	Ile Arg Ile Pro Lys Tyr Lys Asn Asp Gly Ile Gln Asn Tyr Ile His		
5	115	120	125
	Asn Glu Tyr Thr Ile Ile Asn Cys Met Lys Asn Asn Ser Gly Trp Lys		
	130	135	140
10	Ile Ser Ile Arg Gly Asn Arg Ile Ile Trp Thr Leu Ile Asp Ile Asn		
	145	150	155
	Gly Lys Thr Lys Ser Val Phe Phe Glu Tyr Asn Ile Arg Glu Asp Ile		
	165	170	175
15	Ser Glu Tyr Ile Asn Arg Trp Phe Phe Val Thr Ile Thr Asn Asn Leu		
	180	185	190
	Asn Asn Ala Lys Ile Tyr Ile Asn Gly Lys Leu Glu Ser Asn Thr Asp		
20	195	200	205
	Ile Lys Asp Ile Arg Glu Val Ile Ala Asn Gly Glu Ile Ile Phe Lys		
	210	215	220
25	Leu Asp Gly Asp Ile Asp Arg Thr Gln Phe Ile Trp Met Lys Tyr Phe		
	225	230	235
	Ser Ile Phe Asn Thr Glu Leu Ser Gln Ser Asn Ile Glu Glu Arg Tyr		
	245	250	255
30	Lys Ile Gln Ser Tyr Ser Glu Tyr Leu Lys Asp Phe Trp Gly Asn Pro		
	260	265	270
	Leu Met Tyr Asn Lys Glu Tyr Tyr Met Phe Asn Ala Gly Asn Lys Asn		
35	275	280	285
	Ser Tyr Ile Lys Leu Lys Lys Asp Ser Pro Val Gly Glu Ile Leu Thr		
	290	295	300
40	Arg Ser Lys Tyr Asn Gln Asn Ser Lys Tyr Ile Asn Tyr Arg Asp Leu		
	305	310	315
	Tyr Ile Gly Glu Lys Phe Ile Ile Arg Arg Lys Ser Asn Ser Gln Ser		
	325	330	335
45	Ile Asn Asp Asp Ile Val Arg Lys Glu Asp Tyr Ile Tyr Leu Asp Phe		
	340	345	350
	Phe Asn Leu Asn Gln Glu Trp Arg Val Tyr Thr Tyr Lys Tyr Phe Lys		
50	355	360	365
	Lys Glu Glu Glu Lys Leu Phe Leu Ala Pro Ile Ser Asp Ser Asp Glu		
	370	375	380
55	Phe Tyr Asn Thr Ile Gln Ile Lys Glu Tyr Asp Glu Gln Pro Thr Tyr		
	385	390	395
	Ser Cys Gln Leu Leu Phe Lys Lys Asp Glu Glu Ser Thr Asp Glu Ile		
	405	410	415
60	Gly Leu Ile Gly Ile His Arg Phe Tyr Glu Ser Gly Ile Val Phe Glu		
	420	425	430
	Glu Tyr Lys Asp Tyr Phe Cys Ile Ser Lys Trp Tyr Leu Lys Glu Val		
65	435	440	445
	Lys Arg Lys Pro Tyr Asn Leu Lys Leu Gly Cys Asn Trp Gln Phe Ile		
	450	455	460
70	Pro Lys Asp Glu Gly Trp Thr Glu		
	465	470	



(2) INFORMATION FOR SEQ ID NO:47:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 31 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid  
 (A) DESCRIPTION: /desc = "DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

CGCCATGGCT GATACAATAC TAATAGAAAT G 31

(2) INFORMATION FOR SEQ ID NO:48:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 29 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid  
 (A) DESCRIPTION: /desc = "DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

GCAAGCTTTT ATTCAGTCCA CCCTTCATC 29

(2) INFORMATION FOR SEQ ID NO:49:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 3753 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:  
 (A) NAME/KEY: CDS  
 (B) LOCATION: 1..3750

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

ATG CCA ACA ATT AAT AGT TTT AAT TAT AAT GAT CCT GTT AAT AAT AGA 48  
 Met Pro Thr Ile Asn Ser Phe Asn Tyr Asn Asp Pro Val Asn Asn Arg  
 1 5 10 15

ACA ATT TTA TAT ATT AAA CCA GGC GGT TGT CAA CAA TTT TAT AAA TCA 96  
 Thr Ile Leu Tyr Ile Lys Pro Gly Gly Cys Gln Gln Phe Tyr Lys Ser  
 20 25 30

TTT AAT ATT ATG AAA AAT ATT TGG ATA ATT CCA GAG AGA AAT GTA ATT 144  
 Phe Asn Ile Met Lys Asn Ile Trp Ile Ile Pro Glu Arg Asn Val Ile  
 35 40 45

GGT ACA ATT CCC CAA GAT TTT CTT CCG CCT ACT TCA TTG AAA AAT GGA 192  
 Gly Thr Ile Pro Gln Asp Phe Leu Pro Pro Thr Ser Leu Lys Asn Gly  
 50 55 60

GAT AGT AGT TAT TAT GAC CCT AAT TAT TTA CAA AGT GAT CAA GAA AAG 240  
 Asp Ser Ser Tyr Tyr Asp Pro Asn Tyr Leu Gln Ser Asp Gln Glu Lys  
 65 70 75 80

GAT AAA TTT TTA AAA ATA GTC ACA AAA ATA TTT AAT AGA ATA AAT GAT 288  
 Asp Lys Phe Leu Lys Ile Val Thr Lys Ile Phe Asn Arg Ile Asn Asp  
 85 90 95

	AAT CTT TCA GGA AGG ATT TTA TTA GAA GAA CTG TCA AAA GCT AAT CCA Asn Leu Ser Gly Arg Ile Leu Leu Glu Glu Leu Ser Lys Ala Asn Pro	336
	100 105 110	
5	TAT TTA GGA AAT GAT AAT ACT CCA GAT GGT GAC TTC ATT ATT AAT GAT Tyr Leu Gly Asn Asp Asn Thr Pro Asp Gly Asp Phe Ile Ile Asn Asp	384
	115 120 125	
10	GCA TCA GCA GTT CCA ATT CAA TTC TCA AAT GGT AGC CAA AGC ATA CTA Ala Ser Ala Val Pro Ile Gln Phe Ser Asn Gly Ser Gln Ser Ile Leu	432
	130 135 140	
15	TTA CCT AAT GTT ATT ATA ATG GGA GCA GAG CCT GAT TTA TTT GAA ACT Leu Pro Asn Val Ile Ile Met Gly Ala Glu Pro Asp Leu Phe Glu Thr	480
	145 150 155 160	
20	AAC AGT TCC AAT ATT TCT CTA AGA AAT AAT TAT ATG CCA AGC AAT CAC Asn Ser Ser Asn Ile Ser Leu Arg Asn Asn Tyr Met Pro Ser Asn His	528
	165 170 175	
	GGT TTT GGA TCA ATA GCT ATA GTA ACA TTC TCA CCT GAA TAT TCT TTT Gly Phe Gly Ser Ile Ala Ile Val Thr Phe Ser Pro Glu Tyr Ser Phe	576
	180 185 190	
25	AGA TTT AAA GAT AAT AGT ATG AAT GAA TTT ATT CAA GAT CCT GCT CTT Arg Phe Lys Asp Asn Ser Met Asn Glu Phe Ile Gln Asp Pro Ala Leu	624
	195 200 205	
30	ACA TTA ATG CAT GAA TTA ATA CAT TCA TTA CAT GGA CTA TAT GGG GCT Thr Leu Met His Glu Leu Ile His Ser Leu His Gly Leu Tyr Gly Ala	672
	210 215 220	
35	AAA GGG ATT ACT ACA AAG TAT ACT ATA ACA CAA AAA CAA AAT CCC CTA Lys Gly Ile Thr Thr Lys Tyr Thr Ile Thr Gln Lys Gln Asn Pro Leu	720
	225 230 235 240	
40	ATA ACA AAT ATA AGA GGT ACA AAT ATT GAA GAA TTC TTA ACT TTT GGA Ile Thr Asn Ile Arg Gly Thr Asn Ile Glu Glu Phe Leu Thr Phe Gly	768
	245 250 255	
	GGT ACT GAT TTA AAC ATT ATT ACT AGT GCT CAG TCC AAT GAT ATC TAT Gly Thr Asp Leu Asn Ile Ile Thr Ser Ala Gln Ser Asn Asp Ile Tyr	816
	260 265 270	
45	ACT AAT CTT CTA GCT GAT TAT AAA AAA ATA GCG TCT AAA CTT AGC AAA Thr Asn Leu Leu Ala Asp Tyr Lys Lys Ile Ala Ser Lys Leu Ser Lys	864
	275 280 285	
50	GTA CAA GTA TCT AAT CCA CTA CTT AAT CCT TAT AAA GAT GTT TTT GAA Val Gln Val Ser Asn Pro Leu Leu Asn Pro Tyr Lys Asp Val Phe Glu	912
	290 295 300	
55	GCA AAG TAT GGA TTA GAT AAA GAT GCT AGC GGA ATT TAT TCG GTA AAT Ala Lys Tyr Gly Leu Asp Lys Asp Ala Ser Gly Ile Tyr Ser Val Asn	960
	305 310 315 320	
60	ATA AAC AAA TTT AAT GAT ATT TTT AAA AAA TTA TAC AGC TTT ACG GAA Ile Asn Lys Phe Asn Asp Ile Phe Lys Lys Leu Tyr Ser Phe Thr Glu	1008
	325 330 335	
	TTT GAT TTA GCA ACT AAA TTT CAA GTT AAA TGT AGG CAA ACT TAT ATT Phe Asp Leu Ala Thr Lys Phe Gln Val Lys Cys Arg Gln Thr Tyr Ile	1056
	340 345 350	
65	GGA CAG TAT AAA TAC TTC AAA CTT TCA AAC TTG TTA AAT GAT TCT ATT Gly Gln Tyr Lys Tyr Phe Lys Leu Ser Asn Leu Leu Asn Asp Ser Ile	1104
	355 360 365	
70	TAT AAT ATA TCA GAA GGC TAT AAT ATA AAT AAT TTA AAG GTA AAT TTT Tyr Asn Ile Ser Glu Gly Tyr Asn Ile Asn Asn Leu Lys Val Asn Phe	1152

	370	375	380	
5	AGA GGA CAG AAT GCA AAT TTA AAT CCT AGA ATT ATT ACA CCA ATT ACA Arg Gly Gln Asn Ala Asn Leu Asn Pro Arg Ile Ile Thr Pro Ile Thr 385 390 400	1200		
10	GGT AGA GGA CTA GTA AAA AAA ATC ATT AGA TTT TGT AAA AAT ATT GTT Gly Arg Gly Leu Val Lys Lys Ile Ile Arg Phe Cys Lys Asn Ile Val 405 410 415	1248		
15	TCT GTA AAA GGC ATA AGG AAA TCA ATA TGT ATC GAA ATA AAT AAT GGT Ser Val Lys Gly Ile Arg Lys Ser Ile Cys Ile Glu Ile Asn Asn Gly 420 425 430	1296		
20	GAG TTA TTT TTT GTG GCT TCC GAG AAT AGT TAT AAT GAT GAT AAT ATA Glu Leu Phe Phe Val Ala Ser Glu Asn Ser Tyr Asn Asp Asp Asn Ile 435 440 445	1344		
25	AAT ACT CCT AAA GAA ATT GAC GAT ACA GTA ACT TCA AAT AAT AAT TAT Asn Thr Pro Lys Glu Ile Asp Asp Thr Val Thr Ser Asn Asn Asn Tyr 450 455 460	1392		
30	GAA AAT GAT TTA GAT CAG GTT ATT TTA AAT TTT AAT AGT GAA TCA GCA Glu Asn Asp Leu Asp Glu Val Ile Leu Asn Phe Asn Ser Glu Ser Ala 465 470 475 480	1440		
35	CCT GGA CTT TCA GAT GAA AAA TTA AAT TTA ACT ATC CAA AAT GAT GCT Pro Gly Leu Ser Asp Glu Lys Leu Asn Leu Thr Ile Gln Asn Asp Ala 485 490 495	1488		
40	TAT ATA CCA AAA TAT GAT TCT AAT GGA ACA AGT GAT ATA GAA CAA CAT Tyr Ile Pro Lys Tyr Asp Ser Asn Gly Thr Ser Asp Ile Glu Gln His 500 505 510	1536		
45	GAT GTT AAT GAA CTT AAT GTA TTT TTC TAT TTA GAT GCA CAG AAA GTG Asp Val Asn Glu Leu Asn Val Phe Phe Tyr Leu Asp Ala Gln Lys Val 515 520 525	1584		
50	CCC GAA GGT GAA AAT AAT GTC AAT CTC ACC TCT TCA ATT GAT ACA GCA Pro Glu Gly Glu Asn Asn Val Asn Leu Thr Ser Ser Ile Asp Thr Ala 530 535 540	1632		
55	TTA TTA GAA CAA CCT AAA ATA TAT ACA TTT TTT TCA TCA GAA TTT ATT Leu Leu Glu Gln Pro Lys Ile Tyr Thr Phe Phe Ser Ser Glu Phe Ile 545 550 555 560	1680		
60	AAT AAT GTC AAT AAA CCT GTG CAA GCA GCA TTA TTT GTA AGC TGG ATA Asn Asn Val Asn Lys Pro Val Gln Ala Ala Leu Phe Val Ser Trp Ile 565 570 575	1728		
65	CAA CAA GTA TTA GTA GAT TTT ACT ACT GAA GCT AAC CAA AAA AGT ACT Gln Gln Val Leu Val Asp Phe Thr Thr Glu Ala Asn Gln Lys Ser Thr 580 585 590	1776		
70	GTT GAT AAA ATT GCA GAT ATT TCT ATA GTT GTT CCA TAT ATA GGT CTT Val Asp Lys Ile Ala Asp Ile Ser Ile Val Val Pro Tyr Ile Gly Leu 595 600 605	1824		
	GCT TTA AAT ATA GGA AAT GAA GCA CAA AAA GGA AAT TTT AAA GAT GCA Ala Leu Asn Ile Gly Asn Glu Ala Gln Lys Gly Asn Phe Lys Asp Ala 610 615 620	1872		
	CTT GAA TTA TTA GGA GCA GGT ATT TTA TTA GAA TTT GAA CCC GAG CTT Leu Glu Leu Leu Gly Ala Gly Ile Leu Leu Glu Phe Glu Pro Glu Leu 625 630 635 640	1920		
	TTA ATT CCT ACA ATT TTA GTA TTC ACG ATA AAA TCT TTT TTA GGT TCA Leu Ile Pro Thr Ile Leu Val Phe Thr Ile Lys Ser Phe Leu Gly Ser 645 650 655	1968		

	TCT GAT AAT AAA AAT AAA GTT ATT AAA GCA ATA AAT AAT GCA TTG AAA Ser Asp Asn Lys Asn Lys Val Ile Lys Ala Ile Asn Asn Ala Leu Lys 660 665 670	2016
5	GAA AGA GAT GAA AAA TGG AAA GAA GTA TAT AGT TTT ATA GTA TCG AAT Glu Arg Asp Glu Lys Trp Lys Glu Val Tyr Ser Phe Ile Val Ser Asn 675 680 685	2064
10	TGG ATG ACT AAA ATT AAT ACA CAA TTT AAT AAA AGA AAA GAA CAA ATG Trp Met Thr Lys Ile Asn Thr Gln Phe Asn Lys Arg Lys Glu Gln Met 690 695 700	2112
15	TAT CAA GCT TTA CAA AAT CAA GTA AAT GCA CTT AAA GCA ATA ATA GAA Tyr Gln Ala Leu Gln Asn Gln Val Asn Ala Leu Lys Ala Ile Ile Glu 705 710 715 720	2160
20	TCT AAG TAT AAT AGT TAT ACT TTA GAA GAA AAA AAT GAG CTT ACA AAT Ser Lys Tyr Asn Ser Tyr Thr Leu Glu Glu Lys Asn Glu Leu Thr Asn 725 730 735	2208
	AAA TAT GAT ATT GAG CAA ATA GAA AAT GAA CTT AAT CAA AAG GTT TCT Lys Tyr Asp Ile Glu Gln Ile Glu Asn Glu Leu Asn Gln Lys Val Ser 740 745 750	2256
25	ATA GCA ATG AAT AAT ATA GAC AGG TTC TTA ACT GAA AGT TCT ATA TCT Ile Ala Met Asn Asn Ile Asp Arg Phe Leu Thr Glu Ser Ser Ile Ser 755 760 765	2304
30	TAT TTA ATG AAA TTA ATA AAT GAA GTA AAA ATT AAT AAA TTA AGA GAA Tyr Leu Met Lys Leu Ile Asn Glu Val Lys Ile Asn Lys Leu Arg Glu 770 775 780	2352
35	TAT GAT GAA AAT GTT AAA ACG TAT TTA TTA GAT TAT ATT ATA AAA CAT Tyr Asp Glu Asn Val Lys Thr Tyr Leu Leu Asp Tyr Ile Ile Lys His 785 790 795 800	2400
40	GGA TCA ATC TTG GGA GAG AGT CAG CAA GAA CTA AAT TCT ATG GTA ATT Gly Ser Ile Leu Gly Glu Ser Gln Gln Glu Leu Asn Ser Met Val Ile 805 810 815	2448
	GAT ACC CTA AAT AAT AGT ATT CCT TTT AAG CTT TCT TCT TAT ACA GAT Asp Thr Leu Asn Asn Ser Ile Pro Phe Lys Leu Ser Ser Tyr Thr Asp 820 825 830	2496
45	GAT AAA ATT TTA ATT TCA TAT TTT AAT AAG TTC TTT AAG AGA ATT AAA Asp Lys Ile Leu Ile Ser Tyr Phe Asn Lys Phe Phe Lys Arg Ile Lys 835 840 845	2544
50	AGT AGT TCT GTT TTA AAT ATG AGA TAT AAA AAT GAT AAA TAC GTA GAT Ser Ser Ser Val Leu Asn Met Arg Tyr Lys Asn Asp Lys Tyr Val Asp 850 855 860	2592
55	ACT TCA GGA TAT GAT TCA AAT ATA AAT ATT AAT GGA GAT GTA TAT AAA Thr Ser Gly Tyr Asp Ser Asn Ile Asn Ile Asn Gly Asp Val Tyr Lys 865 870 875 880	2640
60	TAT CCA ACT AAT AAA AAT CAA TTT GGA ATA TAT AAT GAT AAA CTT AGT Tyr Pro Thr Asn Lys Asn Gln Phe Gly Ile Tyr Asn Asp Lys Leu Ser 885 890 895	2688
	GAA GTT AAT ATA TCT CAA AAT GAT TAC ATT ATA TAT GAT AAT AAA TAT Glu Val Asn Ile Ser Gln Asn Asp Tyr Ile Ile Tyr Asp Asn Lys Tyr 900 905 910	2736
65	AAA AAT TTT AGT ATT AGT TTT TGG GTA AGA ATT CCT AAC TAT GAT AAT Lys Asn Phe Ser Ile Ser Phe Trp Val Arg Ile Pro Asn Tyr Asp Asn 915 920 925	2784
70	AAG ATA GTA AAT GTT AAT AAT GAA TAC ACT ATA ATA AAT TGT ATG AGG Lys Ile Val Asn Val Asn Asn Glu Tyr Thr Ile Ile Asn Cys Met Arg	2832

	930	935	940	
5	GAT AAT AAT TCA GGA TGG AAA GTA TCT CTT AAT CAT AAT GAA ATA ATT Asp Asn Asn Ser Gly Trp Lys Val Ser Leu Asn His Asn Glu Ile Ile 945 950 955 960	2880		
10	TGG ACA TTG CAA GAT AAT TCA GGA ATT AAT CAA AAA TTA GCA TTT AAC Trp Thr Leu Gln Asp Asn Ser Gly Ile Asn Gln Lys Leu Ala Phe Asn 965 970 975	2928		
15	TAT GGT AAC GCA AAT GGT ATT TCT GAT TAT ATA AAT AAG TGG ATT TTT Tyr Gly Asn Ala Asn Gly Ile Ser Asp Tyr Ile Asn Lys Trp Ile Phe 980 985 990	2976		
20	GTA ACT ATA ACT AAT GAT AGA TTA GGA GAT TCT AAA CTT TAT ATT AAT Val Thr Ile Thr Asn Asp Arg Leu Gly Asp Ser Lys Leu Tyr Ile Asn 995 1000 1005	3024		
25	GGA AAT TTA ATA GAT AAA AAA TCA ATT TTA AAT TTA GGT AAT ATT CAT Gly Asn Leu Ile Asp Lys Lys Ser Ile Leu Asn Leu Gly Asn Ile His 1010 1015 1020	3072		
30	GTT AGT GAC AAT ATA TTA TTT AAA ATA GTT AAT TGT AGT TAT ACA AGA Val Ser Asp Asn Ile Leu Phe Lys Ile Val Asn Cys Ser Tyr Thr Arg 1025 1030 1035 1040	3120		
35	TAT ATT GGT ATT AGA TAT TTT AAT ATT TTT GAT AAA GAA TTA GAT GAA Tyr Ile Gly Ile Arg Tyr Phe Asn Ile Phe Asp Lys Glu Leu Asp Glu 1045 1050 1055	3168		
40	ACA GAA ATT CAA ACT TTA TAT AAC AAT GAA CCT AAT GCA AAT ATT TTA Thr Glu Ile Gln Thr Leu Tyr Asn Asn Glu Pro Asn Ala Asn Ile Leu 1060 1065 1070	3216		
45	AAG GAT TTT TGG GGA AAT TAT TTG CTT TAT GAC AAA GAA TAC TAT TTA Lys Asp Phe Trp Gly Asn Tyr Leu Leu Tyr Asp Lys Glu Tyr Tyr Leu 1075 1080 1085	3264		
50	TTA AAT GTG TTA AAA CCA AAT AAC TTT ATT AAT AGG AGA ACA GAT TCT Leu Asn Val Leu Lys Pro Asn Asn Phe Ile Asn Arg Arg Thr Asp Ser 1090 1095 1100	3312		
55	ACT TTA AGC ATT AAT AAT ATA AGA AGC ACT ATT CTT TTA GCT AAT AGA Thr Leu Ser Ile Asn Asn Ile Arg Ser Thr Ile Leu Leu Ala Asn Arg 1105 1110 1115 1120	3360		
60	TTA TAT AGT GGA ATA AAA GTT AAA ATA CAA AGA GTT AAT AAT AGT AGT Leu Tyr Ser Gly Ile Lys Val Lys Ile Gln Arg Val Asn Asn Ser Ser 1125 1130 1135	3408		
65	ACT AAC GAT AAT CTT GTT AGA AAG AAT GAT CAG GTA TAT ATT AAT TTT Thr Asn Asp Asn Leu Val Arg Lys Asn Asp Gln Val Tyr Ile Asn Phe 1140 1145 1150	3456		
70	GTA GCC AGC AAA ACT CAC TTA CTT CCA TTA TAT GCT GAT ACA GCT ACC Val Ala Ser Lys Thr His Leu Leu Pro Leu Tyr Ala Asp Thr Ala Thr 1155 1160 1165	3504		
75	ACA AAT AAA GAG AAA ACA ATA AAA ATA TCA TCA TCT GGC AAT AGA TTT Thr Asn Lys Glu Lys Thr Ile Lys Ile Ser Ser Ser Gly Asn Arg Phe 1170 1175 1180	3552		
80	AAT CAA GTA GTA GTT ATG AAT TCA GTA GGA TGT ACA ATG AAT TTT AAA Asn Gln Val Val Val Met Asn Ser Val Gly Cys Thr Met Asn Phe Lys 1185 1190 1195 1200	3600		

	AAT AAT AAT GGA AAT AAT ATT GGG TTG TTA GGT TTC AAG GCA GAT ACT Asn Asn Asn Gly Asn Asn Ile Gly Leu Leu Gly Phe Lys Ala Asp Thr 1205 1210 1215	3648
5	GTA GTT GCT AGT ACT TGG TAT TAT ACA CAT ATG AGA GAT AAT ACA AAC Val Val Ala Ser Thr Trp Tyr Tyr Thr His Met Arg Asp Asn Thr Asn 1220 1225 1230	3696
10	AGC AAT GGA TTT TTT TGG AAC TTT ATT TCT GAA GAA CAT GGA TGG CAA Ser Asn Gly Phe Phe Trp Asn Phe Ile Ser Glu Glu His Gly Trp Gln 1235 1240 1245	3744
15	GAA AAA TAA Glu Lys 1250	3753
(2) INFORMATION FOR SEQ ID NO:50:		
20	(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 1250 amino acids (B) TYPE: amino acid (D) TOPOLOGY: linear	
25	(ii) MOLECULE TYPE: protein	
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:	
30	Met Pro Thr Ile Asn Ser Phe Asn Tyr Asn Asp Pro Val Asn Asn Arg 1 5 10 15 Thr Ile Leu Tyr Ile Lys Pro Gly Gly Cys Gln Gln Phe Tyr Lys Ser 20 25 30 Phe Asn Ile Met Lys Asn Ile Trp Ile Ile Pro Glu Arg Asn Val Ile 35 40 45 Gly Thr Ile Pro Gln Asp Phe Leu Pro Pro Thr Ser Leu Lys Asn Gly 50 55 60 Asp Ser Ser Tyr Tyr Asp Pro Asn Tyr Leu Gln Ser Asp Gln Glu Lys 65 70 75 80 Asp Lys Phe Leu Lys Ile Val Thr Lys Ile Phe Asn Arg Ile Asn Asp 85 90 95 Asn Leu Ser Gly Arg Ile Leu Leu Glu Leu Ser Lys Ala Asn Pro 100 105 110 Tyr Leu Gly Asn Asp Asn Thr Pro Asp Gly Asp Phe Ile Ile Asn Asp 115 120 125 Ala Ser Ala Val Pro Ile Gln Phe Ser Asn Gly Ser Gln Ser Ile Leu 130 135 140 Leu Pro Asn Val Ile Ile Met Gly Ala Glu Pro Asp Leu Phe Glu Thr 145 150 155 160 Asn Ser Ser Asn Ile Ser Leu Arg Asn Asn Tyr Met Pro Ser Asn His 165 170 175 Gly Phe Gly Ser Ile Ala Ile Val Thr Phe Ser Pro Glu Tyr Ser Phe 180 185 190 Arg Phe Lys Asp Asn Ser Met Asn Glu Phe Ile Gln Asp Pro Ala Leu 195 200 205 Thr Leu Met His Glu Leu Ile His Ser Leu His Gly Leu Tyr Gly Ala 210 215 220 Lys Gly Ile Thr Thr Lys Tyr Thr Ile Thr Gln Lys Gln Asn Pro Leu	

- 313 -

Ala Leu Asn Ile Gly Asn Glu Ala Gln Lys Gly Asn Phe Lys Asp Ala  
 610 615 620  
 5 Leu Glu Leu Leu Gly Ala Gly Ile Leu Leu Glu Phe Glu Pro Glu Leu  
 625 630 635 640  
 Leu Ile Pro Thr Ile Leu Val Phe Thr Ile Lys Ser Phe Leu Gly Ser  
 645 650 655  
 10 Ser Asp Asn Lys Asn Lys Val Ile Lys Ala Ile Asn Asn Ala Leu Lys  
 660 665 670  
 Glu Arg Asp Glu Lys Trp Lys Glu Val Tyr Ser Phe Ile Val Ser Asn  
 675 680 685  
 15 Trp Met Thr Lys Ile Asn Thr Gln Phe Asn Lys Arg Lys Glu Gln Met  
 690 695 700  
 20 Tyr Gln Ala Leu Gln Asn Gln Val Asn Ala Leu Lys Ala Ile Ile Glu  
 705 710 715 720  
 Ser Lys Tyr Asn Ser Tyr Thr Leu Glu Glu Lys Asn Glu Leu Thr Asn  
 725 730 735  
 25 Lys Tyr Asp Ile Glu Gln Ile Glu Asn Glu Leu Asn Gln Lys Val Ser  
 740 745 750  
 Ile Ala Met Asn Asn Ile Asp Arg Phe Leu Thr Glu Ser Ser Ile Ser  
 755 760 765  
 30 Tyr Leu Met Lys Leu Ile Asn Glu Val Lys Ile Asn Lys Leu Arg Glu  
 770 775 780  
 35 Tyr Asp Glu Asn Val Lys Thr Tyr Leu Leu Asp Tyr Ile Ile Lys His  
 785 790 795 800  
 Gly Ser Ile Leu Gly Glu Ser Gln Gln Glu Leu Asn Ser Met Val Ile  
 805 810 815  
 40 Asp Thr Leu Asn Asn Ser Ile Pro Phe Lys Leu Ser Ser Tyr Thr Asp  
 820 825 830  
 Asp Lys Ile Leu Ile Ser Tyr Phe Asn Lys Phe Phe Lys Arg Ile Lys  
 835 840 845  
 45 Ser Ser Ser Val Leu Asn Met Arg Tyr Lys Asn Asp Lys Tyr Val Asp  
 850 855 860  
 50 Thr Ser Gly Tyr Asp Ser Asn Ile Asn Ile Asn Gly Asp Val Tyr Lys  
 865 870 875 880  
 Tyr Pro Thr Asn Lys Asn Gln Phe Gly Ile Tyr Asn Asp Lys Leu Ser  
 885 890 895  
 55 Glu Val Asn Ile Ser Gln Asn Asp Tyr Ile Ile Tyr Asp Asn Lys Tyr  
 900 905 910  
 Lys Asn Phe Ser Ile Ser Phe Trp Val Arg Ile Pro Asn Tyr Asp Asn  
 915 920 925  
 60 Lys Ile Val Asn Val Asn Asn Glu Tyr Thr Ile Ile Asn Cys Met Arg  
 930 935 940  
 65 Asp Asn Asn Ser Gly Trp Lys Val Ser Leu Asn His Asn Glu Ile Ile  
 945 950 955 960  
 Trp Thr Leu Gln Asp Asn Ser Gly Ile Asn Gln Lys Leu Ala Phe Asn  
 965 970 975  
 70 Tyr Gly Asn Ala Asn Gly Ile Ser Asp Tyr Ile Asn Lys Trp Ile Phe



980 985 990  
 Val Thr Ile Thr Asn Asp Arg Leu Gly Asp Ser Lys Leu Tyr Ile Asn  
 995 1000 1005  
 Gly Asn Leu Ile Asp Lys Lys Ser Ile Leu Asn Leu Gly Asn Ile His  
 1010 1015 1020  
 Val Ser Asp Asn Ile Leu Phe Lys Ile Val Asn Cys Ser Tyr Thr Arg  
 1025 1030 1035 1040  
 Tyr Ile Gly Ile Arg Tyr Phe Asn Ile Phe Asp Lys Glu Leu Asp Glu  
 1045 1050 1055  
 Thr Glu Ile Gln Thr Leu Tyr Asn Asn Glu Pro Asn Ala Asn Ile Leu  
 1060 1065 1070  
 Lys Asp Phe Trp Gly Asn Tyr Leu Leu Tyr Asp Lys Glu Tyr Tyr Leu  
 1075 1080 1085  
 Leu Asn Val Leu Lys Pro Asn Asn Phe Ile Asn Arg Arg Thr Asp Ser  
 1090 1095 1100  
 Thr Leu Ser Ile Asn Asn Ile Arg Ser Thr Ile Leu Leu Ala Asn Arg  
 1105 1110 1115 1120  
 Leu Tyr Ser Gly Ile Lys Val Lys Ile Gln Arg Val Asn Asn Ser Ser  
 1125 1130 1135  
 Thr Asn Asp Asn Leu Val Arg Lys Asn Asp Gln Val Tyr Ile Asn Phe  
 1140 1145 1150  
 Val Ala Ser Lys Thr His Leu Leu Pro Leu Tyr Ala Asp Thr Ala Thr  
 1155 1160 1165  
 Thr Asn Lys Glu Lys Thr Ile Lys Ile Ser Ser Ser Gly Asn Arg Phe  
 1170 1175 1180  
 Asn Gln Val Val Val Met Asn Ser Val Gly Cys Thr Met Asn Phe Lys  
 1185 1190 1195 1200  
 Asn Asn Asn Gly Asn Asn Ile Gly Leu Leu Gly Phe Lys Ala Asp Thr  
 1205 1210 1215  
 Val Val Ala Ser Thr Trp Tyr Tyr Thr His Met Arg Asp Asn Thr Asn  
 1220 1225 1230  
 Ser Asn Gly Phe Phe Trp Asn Phe Ile Ser Glu Glu His Gly Trp Gln  
 1235 1240 1245  
 Glu Lys  
 1250

## (2) INFORMATION FOR SEQ ID NO:51:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 3759 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

- (ix) FEATURE:  
 (A) NAME/KEY: CDS  
 (B) LOCATION: 1..3756

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

ATG CCA AAA ATT AAT AGT TTT AAT TAT AAT GAT CCT GTT AAT GAT AGA

	Met	Pro	Lys	Ile	Asn	Ser	Phe	Asn	Tyr	Asn	Asp	Pro	Val	Asn	Asp	Arg	
	1				5					10					15		
5	ACA	ATT	TTA	TAT	ATT	AAA	CCA	GGC	GGT	TGT	CAA	GAA	TTT	TAT	AAA	TCA	96
	Thr	Ile	Leu	Tyr	Ile	Lys	Pro	Gly	Gly	Cys	Gln	Glu	Phe	Tyr	Lys	Ser	
				20					25					30			
10	TTT	AAT	ATT	ATG	AAA	AAT	ATT	TGG	ATA	ATT	CCA	GAG	AGA	AAT	GTA	ATT	144
	Phe	Asn	Ile	Met	Lys	Asn	Ile	Trp	Ile	Ile	Pro	Glu	Arg	Asn	Val	Ile	
				35				40					45				
15	GGT	ACA	ACC	CCC	CAA	GAT	TTT	CAT	CCG	CCT	ACT	TCA	TTA	AAA	AAT	GGA	192
	Gly	Thr	Thr	Pro	Gln	Asp	Phe	His	Pro	Pro	Thr	Ser	Leu	Lys	Asn	Gly	
				50			55					60					
20	GAT	AGT	AGT	TAT	TAT	GAC	CCT	AAT	TAT	TTA	CAA	AGT	GAT	GAA	GAA	AAG	240
	Asp	Ser	Ser	Tyr	Tyr	Asp	Pro	Asn	Tyr	Leu	Gln	Ser	Asp	Glu	Glu	Lys	
						70				75				80			
25	GAT	AGA	TTT	TTA	AAA	ATA	GTC	ACA	AAA	ATA	TTT	AAT	AGA	ATA	AAT	AAT	288
	Asp	Arg	Phe	Leu	Lys	Ile	Val	Thr	Lys	Ile	Phe	Asn	Arg	Ile	Asn	Asn	
					85					90				95			
30	AAT	CTT	TCA	GGA	GGG	ATT	TTA	TTA	GAA	GAA	CTG	TCA	AAA	GCT	AAT	CCA	336
	Asn	Leu	Ser	Gly	Gly	Ile	Leu	Leu	Glu	Glu	Leu	Ser	Lys	Ala	Asn	Pro	
				100					105					110			
35	TAT	TTA	GGG	AAT	GAT	AAT	ACT	CCA	GAT	AAT	CAA	TTC	CAT	ATT	GGT	GAT	384
	Tyr	Leu	Gly	Asn	Asp	Asn	Thr	Pro	Asp	Asn	Gln	Phe	His	Ile	Gly	Asp	
				115				120					125				
40	GCA	TCA	GCA	GTT	GAG	ATT	AAA	TTC	TCA	AAT	GGT	AGC	CAA	GAC	ATA	CTA	432
	Ala	Ser	Ala	Val	Glu	Ile	Lys	Phe	Ser	Asn	Gly	Ser	Gln	Asp	Ile	Leu	
				130			135					140					
45	TTA	CCT	AAT	GTT	ATT	ATA	ATG	GGA	GCA	GAG	CCT	GAT	TTA	TTT	GAA	ACT	480
	Leu	Pro	Asn	Val	Ile	Ile	Met	Gly	Ala	Glu	Pro	Asp	Leu	Phe	Glu	Thr	
						150					155				160		
50	AAC	AGT	TCC	AAT	ATT	TCT	CTA	AGA	AAT	AAT	TAT	ATG	CCA	AGC	AAT	CAC	528
	Asn	Ser	Ser	Asn	Ile	Ser	Leu	Arg	Asn	Asn	Tyr	Met	Pro	Ser	Asn	His	
					165					170					175		
55	GGT	TTT	GGA	TCA	ATA	GCT	ATA	GTA	ACA	TTC	TCA	CCT	GAA	TAT	TCT	TTT	576
	Gly	Phe	Gly	Ser	Ile	Ala	Ile	Val	Thr	Phe	Ser	Pro	Glu	Tyr	Ser	Phe	
				180					185					190			
60	AGA	TTT	AAT	GAT	AAT	AGT	ATG	AAT	GAA	TTT	ATT	CAA	GAT	CCT	GCT	CTT	624
	Arg	Phe	Asn	Asp	Asn	Ser	Met	Asn	Glu	Phe	Ile	Gln	Asp	Pro	Ala	Leu	
				195				200					205				
65	ACA	TTA	ATG	CAT	GAA	TTA	ATA	CAT	TCA	TTA	CAT	GGA	CTA	TAT	GGG	GCT	672
	Thr	Leu	Met	His	Glu	Leu	Ile	His	Ser	Leu	His	Gly	Leu	Tyr	Gly	Ala	
				210			215					220					
70	AAA	GGG	ATT	ACT	ACA	AAG	TAT	ACT	ATA	ACA	CAA	AAA	CAA	AAT	CCC	CTA	720
	Lys	Gly	Ile	Thr	Thr	Lys	Tyr	Thr	Ile	Thr	Gln	Lys	Gln	Asn	Pro	Leu	
						230					235				240		
75	ATA	ACA	AAT	ATA	AGA	GGT	ACA	AAT	ATT	GAA	GAA	TTC	TTA	ACT	TTT	GGA	768
	Ile	Thr	Asn	Ile	Arg	Gly	Thr	Asn	Ile	Glu	Glu	Phe	Leu	Thr	Phe	Gly	
					245					250					255		
80	GGT	ACT	GAT	TTA	AAC	ATT	ATT	ACT	AGT	GCT	CAG	TCC	AAT	GAT	ATC	TAT	816
	Gly	Thr	Asp	Leu	Asn	Ile	Ile	Thr	Ser	Ala	Gln	Ser	Asn	Asp	Ile	Tyr	
				260					265					270			
85	ACT	AAT	CTT	CTA	GCT	GAT	TAT	AAA	AAA	ATA	GCG	TCT	AAA	CTT	AGC	AAA	864
	Thr	Asn	Leu	Ala	Asp	Tyr	Lys	Lys	Ile	Ala	Ser	Lys	Leu	Ser	Lys		
				275				280					285				

	GTA CAA GTA TCT AAT CCA CTA CTT AAT CCT TAT AAA GAT GTT TTT GAA Val Gln Val Ser Asn Pro Leu Leu Asn Pro Tyr Lys Asp Val Phe Glu 290 295 300	912
5	GCA AAG TAT GGA TTA GAT AAA GAT GCT AGC GGA ATT TAT TCG GTA AAT Ala Lys Tyr Gly Leu Asp Lys Asp Ala Ser Gly Ile Tyr Ser Val Asn 305 310 315 320	960
10	ATA AAC AAA TTT AAT GAT ATT TTT AAA AAA TTA TAC AGC TTT ACG GAA Ile Asn Lys Phe Asn Asp Ile Phe Lys Lys Leu Tyr Ser Phe Thr Glu 325 330 335	1008
15	TTT GAT TTA GCA ACT AAA TTT CAA GTT AAA TGT AGG CAA ACT TAT ATT Phe Asp Leu Ala Thr Lys Phe Gln Val Lys Cys Arg Gln Thr Tyr Ile 340 345 350	1056
20	GGA CAG TAT AAA TAC TTC AAA CTT TCA AAC TTG TTA AAT GAT TCT ATT Gly Gln Tyr Lys Tyr Phe Lys Leu Ser Asn Leu Leu Asn Asp Ser Ile 355 360 365	1104
	TAT AAT ATA TCA GAA GGC TAT AAT ATA AAT AAT TTA AAG GTA AAT TTT Tyr Asn Ile Ser Glu Gly Tyr Asn Ile Asn Asn Leu Lys Val Asn Phe 370 375 380	1152
25	AGA GGA CAG AAT GCA AAT TTA AAT CCT AGA ATT ATT ACA CCA ATT ACA Arg Gly Gln Asn Ala Asn Leu Asn Pro Arg Ile Ile Thr Pro Ile Thr 385 390 395 400	1200
30	GGT AGA GGA CTA GTA AAA AAA ATC ATT AGA TTT TGT AAA AAT ATT GTT Gly Arg Gly Leu Val Lys Lys Ile Ile Arg Phe Cys Lys Asn Ile Val 405 410 415	1248
35	TCT GTA AAA GGC ATA AGG AAA TCA ATA TGT ATC GAA ATA AAT AAT GGT Ser Val Lys Gly Ile Arg Lys Ser Ile Cys Ile Glu Ile Asn Asn Gly 420 425 430	1296
40	GAG TTA TTT TTT GTG GCT TCC GAG AAT AGT TAT AAT GAT GAT AAT ATA Glu Leu Phe Phe Val Ala Ser Glu Asn Ser Tyr Asn Asp Asp Asn Ile 435 440 445	1344
	AAT ACT CCT AAA GAA ATT GAC GAT ACA GTA ACT TCA AAT AAT AAT TAT Asn Thr Pro Lys Glu Ile Asp Asp Thr Val Thr Ser Asn Asn Asn Tyr 450 455 460	1392
45	GAA AAT GAT TTA GAT CAG GTT ATT TTA AAT TTT AAT AGT GAA TCA GCA Glu Asn Asp Leu Asp Gln Val Ile Leu Asn Phe Asn Ser Glu Ser Ala 465 470 475 480	1440
50	CCT GGA CTT TCA GAT GAA AAA TTA AAT TTA ACT ATC CAA AAT GAT GCT Pro Gly Leu Ser Asp Glu Lys Leu Asn Leu Thr Ile Gln Asn Asp Ala 485 490 495	1488
55	TAT ATA CCA AAA TAT GAT TCT AAT GGA ACA AGT GAT ATA GAA CAA CAT Tyr Ile Pro Lys Tyr Asp Ser Asn Gly Thr Ser Asp Ile Glu Gln His 500 505 510	1536
60	GAT GTT AAT GAA CTT AAT GTA TTT TTC TAT TTA GAT GCA CAG AAA GTG Asp Val Asn Glu Leu Asn Val Phe Phe Tyr Leu Asp Ala Gln Lys Val 515 520 525	1584
	CCC GAA GGT GAA AAT AAT GTC AAT CTC ACC TCT TCA ATT GAT ACA GCA Pro Glu Gly Glu Asn Asn Val Asn Leu Thr Ser Ser Ile Asp Thr Ala 530 535 540	1632
65	TTA TTA GAA CAA CCT AAA ATA TAT ACA TTT TTT TCA TCA GAA TTT ATT Leu Leu Glu Gln Pro Lys Ile Tyr Thr Phe Phe Ser Ser Glu Phe Ile 545 550 555 560	1680
70	AAT AAT GTC AAT AAA CCT GTG CAA GCA GCA TTA TTT GTA AGC TGG ATA Asn Asn Val Asn Lys Pro Val Gln Ala Ala Leu Phe Val Ser Trp Ile	1728

	565	570	575	
5	CAA CAA GTG TTA GTA GAT TTT ACT ACT GAA GCT AAC CAA AAA AGT ACT Gln Gln Val Leu Val Asp Phe Thr Thr Glu Ala Asn Gln Lys Ser Thr 580 585 590			1776
10	GTT GAT AAA ATT GCA GAT ATT TCT ATA GTT GTT CCA TAT ATA GGT CTT Val Asp Lys Ile Ala Asp Ile Ser Ile Val Val Pro Tyr Ile Gly Leu 595 600 605			1824
15	GCT TTA AAT ATA GGA AAT GAA GCA CAA AAA GGA AAT TTT AAA GAT GCA Ala Leu Asn Ile Gly Asn Glu Ala Gln Lys Gly Asn Phe Lys Asp Ala 610 615 620			1872
20	CTT GAA TTA TTA GGA GCA GGT ATT TTA TTA GAA TTT GAA CCC GAG CTT Leu Glu Leu Leu Gly Ala Gly Ile Leu Leu Glu Phe Glu Pro Glu Leu 625 630 635 640			1920
25	TTA ATT CCT ACA ATT TTA GTA TTC ACG ATA AAA TCT TTT TTA GGT TCA Leu Ile Pro Thr Ile Leu Val Phe Thr Ile Lys Ser Phe Leu Gly Ser 645 650 655			1968
30	TCT GAT AAT AAA AAT AAA GTT ATT AAA GCA ATA AAT AAT GCA TTG AAA Ser Asp Asn Lys Asn Lys Val Ile Lys Ala Ile Asn Asn Ala Leu Lys 660 665 670			2016
35	GAA AGA GAT GAA AAA TGG AAA GAA GTA TAT AGT TTT ATA GTA TCG AAT Glu Arg Asp Glu Lys Trp Lys Glu Val Tyr Ser Phe Ile Val Ser Asn 675 680 685			2064
40	TGG ATG ACT AAA ATT AAT ACA CAA TTT AAT AAA AGA AAA GAA CAA ATG Trp Met Thr Lys Ile Asn Thr Gln Phe Asn Lys Arg Lys Glu Gln Met 690 695 700			2112
45	TAT CAA GCT TTA CAA AAT CAA GTA AAT GCA ATT AAA ACA ATA ATA GAA Tyr Gln Ala Leu Gln Asn Gln Val Asn Ala Ile Lys Thr Ile Ile Glu 705 710 715 720			2160
50	TCT AAG TAT AAT AGT TAT ACT TTA GAG GAA AAA AAT GAG CTT ACA AAT Ser Lys Tyr Asn Ser Tyr Thr Leu Glu Glu Lys Asn Glu Leu Thr Asn 725 730 735			2208
55	AAA TAT GAT ATT AAG CAA ATA GAA AAT GAA CTT AAT CAA AAG GTT TCT Lys Tyr Asp Ile Lys Gln Ile Glu Asn Glu Leu Asn Gln Lys Val Ser 740 745 750			2256
60	ATA GCA ATG AAT AAT ATA GAC AGG TTC TTA ACT GAA AGT TCT ATA TCC Ile Ala Met Asn Asn Ile Asp Arg Phe Leu Thr Glu Ser Ser Ile Ser 755 760 765			2304
65	TAT TTA ATG AAA TTA ATA AAT GAA GTA AAA ATT AAT AAA TTA AGA GAA Tyr Leu Met Lys Leu Ile Asn Glu Val Lys Ile Asn Lys Leu Arg Glu 770 775 780			2352
70	TAT GAT GAG AAT GTC AAA ACG TAT TTA TTG AAT TAT ATT ATA CAA CAT Tyr Asp Glu Asn Val Lys Thr Tyr Leu Leu Asn Tyr Ile Ile Gln His 785 790 795 800			2400
75	GGA TCA ATC TTG GGA GAG AGT CAG CAA GAA CTA AAT TCT ATG GTA ACT Gly Ser Ile Leu Gly Glu Ser Gln Gln Glu Leu Asn Ser Met Val Thr 805 810 815			2448
80	GAT ACC CTA AAT AAT AGT ATT CCT TTT AAG CTT TCT TCT TAT ACA GAT Asp Thr Leu Asn Asn Ser Ile Pro Phe Lys Leu Ser Ser Tyr Thr Asp 820 825 830			2496
85	GAT AAA ATT TTA ATT TCA TAT TTT AAT AAA TTC TTT AAG AGA ATT AAA Asp Lys Ile Leu Ile Ser Tyr Phe Asn Lys Phe Phe Lys Arg Ile Lys 835 840 845			2544

	AGT AGT TCA GTT TTA AAT ATG AGA TAT AAA AAT GAT AAA TAC GTA GAT	2592
	Ser Ser Ser Val Leu Asn Met Arg Tyr Lys Asn Asp Lys Tyr Val Asp	
	850 855 860	
5	ACT TCA GGA TAT GAT TCA AAT ATA AAT ATT AAT GGA GAT GTA TAT AAA	2640
	Thr Ser Gly Tyr Asp Ser Asn Ile Asn Ile Asn Gly Asp Val Tyr Lys	
	865 870 875 880	
10	TAT CCA ACT AAT AAA AAT CAA TTT GGA ATA TAT AAT GAT AAA CTT AGT	2688
	Tyr Pro Thr Asn Lys Asn Gln Phe Gly Ile Tyr Asn Asp Lys Leu Ser	
	885 890 895	
15	GAA GTT AAT ATA TCT CAA AAT GAT TAC ATT ATA TAT GAT AAT AAA TAT	2736
	Glu Val Asn Ile Ser Gln Asn Asp Tyr Ile Ile Tyr Asp Asn Lys Tyr	
	900 905 910	
20	AAA AAT TTT AGT ATT AGT TTT TGG GTA AGA ATT CCT AAC TAT GAT AAT	2784
	Lys Asn Phe Ser Ile Ser Phe Trp Val Arg Ile Pro Asn Tyr Asp Asn	
	915 920 925	
25	AAG ATA GTA AAT GTT AAT AAT GAA TAC ACT ATA ATA AAT TGT ATG AGA	2832
	Lys Ile Val Asn Val Asn Asn Glu Tyr Thr Ile Ile Asn Cys Met Arg	
	930 935 940	
30	GAT AAT AAT TCA GGA TGG AAA GTA TCT CTT AAT CAT AAT GAA ATA ATT	2880
	Asp Asn Asn Ser Gly Trp Lys Val Ser Leu Asn His Asn Glu Ile Ile	
	945 950 955 960	
35	TGG ACA TTG CAA GAT AAT GCA GGA ATT AAT CAA AAA TTA GCA TTT AAC	2928
	Trp Thr Leu Gln Asp Asn Ala Gly Ile Asn Gln Lys Leu Ala Phe Asn	
	965 970 975	
40	TAT GGT AAC GCA AAT GGT ATT TCT GAT TAT ATA AAT AAG TGG ATT TTT	2976
	Tyr Gly Asn Ala Asn Gly Ile Ser Asp Tyr Ile Asn Lys Trp Ile Phe	
	980 985 990	
45	GTA ACT ATA ACT AAT GAT AGA TTA GGA GAT TCT AAA CTT TAT ATT AAT	3024
	Val Thr Ile Thr Asn Asp Arg Leu Gly Asp Ser Lys Leu Tyr Ile Asn	
	995 1000 1005	
50	GGA AAT TTA ATA GAT CAA AAA TCA ATT TTA AAT TTA GGT AAT ATT CAT	3072
	Gly Asn Leu Ile Asp Gln Lys Ser Ile Leu Asn Leu Gly Asn Ile His	
	1010 1015 1020	
55	GTT AGT GAC AAT ATA TTA TTT AAA ATA GTT AAT TGT AGT TAT ACA AGA	3120
	Val Ser Asp Asn Ile Leu Phe Lys Ile Val Asn Cys Ser Tyr Thr Arg	
	1025 1030 1035 1040	
60	TAT ATT GGT ATT AGA TAT TTT AAT ATT TTT GAT AAA GAA TTA GAT GAA	3168
	Tyr Ile Gly Ile Arg Tyr Phe Asn Ile Phe Asp Lys Glu Leu Asp Glu	
	1045 1050 1055	
65	ACA GAA ATT CAA ACT TTA TAT AGC AAT GAA CCT AAT ACA AAT ATT TTG	3216
	Thr Glu Ile Gln Thr Leu Tyr Ser Asn Glu Pro Asn Thr Asn Ile Leu	
	1060 1065 1070	
70	AAG GAT TTT TGG GGA AAT TAT TTG CTT TAT GAC AAA GAA TAC TAT TTA	3264
	Lys Asp Phe Trp Gly Asn Tyr Leu Leu Tyr Asp Lys Glu Tyr Tyr Leu	
	1075 1080 1085	
75	TTA AAT GTG TTA AAA CCA AAT AAC TTT ATT GAT AGG AGA AAA GAT TCT	3312
	Leu Asn Val Leu Lys Pro Asn Asn Phe Ile Asp Arg Arg Lys Asp Ser	
	1090 1095 1100	
80	ACT TTA AGC ATT AAT AAT ATA AGA AGC ACT ATT CTT TTA GCT AAT AGA	3360
	Thr Leu Ser Ile Asn Asn Ile Arg Ser Thr Ile Leu Leu Ala Asn Arg	
	1105 1110 1115 1120	
85	TTA TAT AGT GGA ATA AAA GTT AAA ATA CAA AGA GTT AAT AAT AGT AGT	3408
	Leu Tyr Ser Gly Ile Lys Val Lys Ile Gln Arg Val Asn Asn Ser Ser	

	1125	1130	1135	
5	ACT AAC GAT AAT CTT GTT AGA AAG AAT GAT CAG GTA TAT ATT AAT TTT Thr Asn Asp Asn Leu Val Arg Lys Asn Asp Gln Val Tyr Ile Asn Phe 1140 1145 1150	3456		
10	GTA GCC AGC AAA ACT CAC TTA TTT CCA TTA TAT GCT GAT ACA GCT ACC Val Ala Ser Lys Thr His Leu Phe Pro Leu Tyr Ala Asp Thr Ala Thr 1155 1160 1165	3504		
15	ACA AAT AAA GAG AAA ACA ATA AAA ATA TCA TCA TCT GGC AAT AGA TTT Thr Asn Lys Glu Lys Thr Ile Lys Ile Ser Ser Ser Gly Asn Arg Phe 1170 1175 1180	3552		
20	AAT CAA GTA GTA GTT ATG AAT TCA GTA GGA AAT AAT TGT ACA ATG AAT Asn Gln Val Val Val Met Asn Ser Val Gly Asn Asn Cys Thr Met Asn 1185 1190 1195 1200	3600		
25	TTT AAA AAT AAT AAT GGA AAT AAT ATT GGG TTG TTA GGT TTC AAG GCA Phe Lys Asn Asn Asn Gly Asn Asn Ile Gly Leu Leu Gly Phe Lys Ala 1205 1210 1215	3648		
30	GAT ACT GTA GTT GCT AGT ACT TGG TAT TAT ACA CAT ATG AGA GAT CAT Asp Thr Val Val Ala Ser Thr Trp Tyr Tyr Thr His Met Arg Asp His 1220 1225 1230	3696		
35	ACA AAC AGC AAT GGA TGT TTT TGG AAC TTT ATT TCT GAA GAA CAT GGA Thr Asn Ser Asn Gly Cys Phe Trp Asn Phe Ile Ser Glu Glu His Gly 1235 1240 1245	3744		
40	TGG CAA GAA AAA TAA Trp Gln Glu Lys 1250	3759		
45	(2) INFORMATION FOR SEQ ID NO:52:			
50	(i) SEQUENCE CHARACTERISTICS:			
	(A) LENGTH: 1252 amino acids			
	(B) TYPE: amino acid			
	(D) TOPOLOGY: linear			
55	(ii) MOLECULE TYPE: protein			
60	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:			
65	Met Pro Lys Ile Asn Ser Phe Asn Tyr Asn Asp Pro Val Asn Asp Arg 1 5 10 15			
70	Thr Ile Leu Tyr Ile Lys Pro Gly Gly Cys Gln Glu Phe Tyr Lys Ser 20 25 30			
75	Phe Asn Ile Met Lys Asn Ile Trp Ile Ile Pro Glu Arg Asn Val Ile 35 40 45			
80	Gly Thr Thr Pro Gln Asp Phe His Pro Pro Thr Ser Leu Lys Asn Gly 50 55 60			
85	Asp Ser Ser Tyr Tyr Asp Pro Asn Tyr Leu Gln Ser Asp Glu Glu Lys 65 70 75 80			
90	Asp Arg Phe Leu Lys Ile Val Thr Lys Ile Phe Asn Arg Ile Asn Asn 85 90 95			
95	Asn Leu Ser Gly Gly Ile Leu Leu Glu Leu Ser Lys Ala Asn Pro 100 105 110			
100	Tyr Leu Gly Asn Asp Asn Thr Pro Asp Asn Gln Phe His Ile Gly Asp 115 120 125			
105	Ala Ser Ala Val Glu Ile Lys Phe Ser Asn Gly Ser Gln Asp Ile Leu			

	130	135	140
5	Leu 145	Pro Asn Val Ile 150	Ile Met Gly Ala Glu 155
	Asn Ser Ser Asn 165	Ile Ser Leu Arg Asn 170	Asp Tyr Met Pro Ser Asn 175
10	Gly Phe Gly 180	Ser Ile Ala Ile Val 185	Thr Phe Ser Pro Glu Tyr Ser Phe 190
	Arg Phe 195	Asn Asp Asn Ser Met 200	Glu Phe Ile Gln Asp Pro Ala Leu 205
15	Thr 210	Leu Met His Glu Leu 215	Ile His Ser Leu His Gly 220
	Lys 225	Gly Ile Thr Thr 230	Lys Tyr Thr Ile Thr Gln Lys Gln Asn Pro Leu 240
20	Ile Thr Asn 245	Ile Arg Gly Thr Asn 250	Ile Glu Glu Phe Leu Thr Phe Gly 255
25	Gly Thr Asp 260	Leu Asn Ile Ile Thr Ser 265	Ala Gln Ser Asn Asp Ile Tyr 270
	Thr Asn 275	Leu Leu Ala Asp Tyr 280	Lys Lys Ile Ala Ser Lys Leu Ser Lys 285
30	Val 290	Gln Val Ser Asn Pro 295	Leu Leu Asn Pro Tyr Lys Asp Val Phe Glu 300
	Ala 305	Lys Tyr Gly Leu Asp 310	Lys Asp Ala Ser Gly Ile Tyr Ser Val Asn 320
35	Ile Asn Lys Phe 325	Asn Asp Ile Phe Lys 330	Lys Leu Tyr Ser Phe Thr Glu 335
40	Phe Asp Leu 340	Ala Thr Lys Phe Gln 345	Val Lys Cys Arg Gln Thr Tyr Ile 350
	Gly Gln Tyr 355	Lys Tyr Phe Lys Leu Ser Asn 360	Leu Leu Asn Asp Ser Ile 365
45	Tyr Asn 370	Ile Ser Glu Gly Tyr 375	Asn Ile Asn Asn Leu Lys Val Asn Phe 380
	Arg Gly Gln Asn Ala 385	Asn Leu Asn Pro Arg 390	Ile Ile Thr Pro Ile Thr 400
50	Gly Arg Gly Leu 405	Val Lys Lys Ile Ile Arg 410	Phe Cys Lys Asn Ile Val 415
55	Ser Val Lys 420	Gly Ile Arg Lys Ser 425	Ile Cys Ile Glu Ile Asn Asn Gly 430
	Glu Leu Phe 435	Phe Val Ala Ser Glu 440	Asn Ser Tyr Asn Asp Asp Asn Ile 445
60	Asn Thr 450	Pro Lys Glu Ile Asp 455	Asp Thr Val Thr Ser Asn Asn Asn Tyr 460
	Glu Asn Asp Leu Asp 465	Gln Val Ile Leu Asn 470	Phe Asn Ser Glu Ser Ala 475 480
65	Pro Gly Leu Ser 485	Asp Glu Lys Leu Asn 490	Leu Thr Ile Gln Asn Asp Ala 495
70	Tyr Ile Pro 500	Lys Tyr Asp Ser Asn Gly 505	Thr Ser Asp Ile Glu Gln His 510

Asp Val Asn Glu Leu Asn Val Phe Phe Tyr Leu Asp Ala Gln Lys Val  
 515 520 525  
 5 Pro Glu Gly Glu Asn Asn Val Asn Leu Thr Ser Ser Ile Asp Thr Ala  
 530 535 540  
 Leu Leu Glu Gln Pro Lys Ile Tyr Thr Phe Phe Ser Ser Glu Phe Ile  
 545 550 555 560  
 10 Asn Asn Val Asn Lys Pro Val Gln Ala Ala Leu Phe Val Ser Trp Ile  
 565 570 575  
 Gln Gln Val Leu Val Asp Phe Thr Thr Glu Ala Asn Gln Lys Ser Thr  
 580 585 590  
 15 Val Asp Lys Ile Ala Asp Ile Ser Ile Val Val Pro Tyr Ile Gly Leu  
 595 600 605  
 20 Ala Leu Asn Ile Gly Asn Glu Ala Gln Lys Gly Asn Phe Lys Asp Ala  
 610 615 620  
 Leu Glu Leu Leu Gly Ala Gly Ile Leu Leu Glu Phe Glu Pro Glu Leu  
 625 630 635 640  
 25 Leu Ile Pro Thr Ile Leu Val Phe Thr Ile Lys Ser Phe Leu Gly Ser  
 645 650 655  
 Ser Asp Asn Lys Asn Lys Val Ile Lys Ala Ile Asn Asn Ala Leu Lys  
 660 665 670  
 30 Glu Arg Asp Glu Lys Trp Lys Glu Val Tyr Ser Phe Ile Val Ser Asn  
 675 680 685  
 35 Trp Met Thr Lys Ile Asn Thr Gln Phe Asn Lys Arg Lys Glu Gln Met  
 690 695 700  
 Tyr Gln Ala Leu Gln Asn Gln Val Asn Ala Ile Lys Thr Ile Ile Glu  
 705 710 715 720  
 40 Ser Lys Tyr Asn Ser Tyr Thr Leu Glu Glu Lys Asn Glu Leu Thr Asn  
 725 730 735  
 Lys Tyr Asp Ile Lys Gln Ile Glu Asn Glu Leu Asn Gln Lys Val Ser  
 740 745 750  
 45 Ile Ala Met Asn Asn Ile Asp Arg Phe Leu Thr Glu Ser Ser Ile Ser  
 755 760 765  
 50 Tyr Leu Met Lys Leu Ile Asn Glu Val Lys Ile Asn Lys Leu Arg Glu  
 770 775 780  
 Tyr Asp Glu Asn Val Lys Thr Tyr Leu Leu Asn Tyr Ile Ile Gln His  
 785 790 795 800  
 55 Gly Ser Ile Leu Gly Glu Ser Gln Gln Glu Leu Asn Ser Met Val Thr  
 805 810 815  
 Asp Thr Leu Asn Asn Ser Ile Pro Phe Lys Leu Ser Ser Tyr Thr Asp  
 820 825 830  
 60 Asp Lys Ile Leu Ile Ser Tyr Phe Asn Lys Phe Phe Lys Arg Ile Lys  
 835 840 845  
 Ser Ser Ser Val Leu Asn Met Arg Tyr Lys Asn Asp Lys Tyr Val Asp  
 850 855 860  
 65 Thr Ser Gly Tyr Asp Ser Asn Ile Asn Ile Asn Gly Asp Val Tyr Lys  
 865 870 875 880  
 70 Tyr Pro Thr Asn Lys Asn Gln Phe Gly Ile Tyr Asn Asp Lys Leu Ser



	885	890	895
5	Glu Val Asn Ile Ser Gln Asn Asp Tyr Ile Ile Tyr Asp Asn Lys Tyr 900 905 910		
	Lys Asn Phe Ser Ile Ser Phe Trp Val Arg Ile Pro Asn Tyr Asp Asn 915 920 925		
10	Lys Ile Val Asn Val Asn Asn Glu Tyr Thr Ile Ile Asn Cys Met Arg 930 935 940		
	Asp Asn Asn Ser Gly Trp Lys Val Ser Leu Asn His Asn Glu Ile Ile 945 950 955 960		
15	Trp Thr Leu Gln Asp Asn Ala Gly Ile Asn Gln Lys Leu Ala Phe Asn 965 970 975		
	Tyr Gly Asn Ala Asn Gly Ile Ser Asp Tyr Ile Asn Lys Trp Ile Phe 980 985 990		
20	Val Thr Ile Thr Asn Asp Arg Leu Gly Asp Ser Lys Leu Tyr Ile Asn 995 1000 1005		
	Gly Asn Leu Ile Asp Gln Lys Ser Ile Leu Asn Leu Gly Asn Ile His 1010 1015 1020		
	Val Ser Asp Asn Ile Leu Phe Lys Ile Val Asn Cys Ser Tyr Thr Arg 1025 1030 1035 1040		
30	Tyr Ile Gly Ile Arg Tyr Phe Asn Ile Phe Asp Lys Glu Leu Asp Glu 1045 1050 1055		
	Thr Glu Ile Gln Thr Leu Tyr Ser Asn Glu Pro Asn Thr Asn Ile Leu 1060 1065 1070		
35	Lys Asp Phe Trp Gly Asn Tyr Leu Leu Tyr Asp Lys Glu Tyr Tyr Leu 1075 1080 1085		
	Leu Asn Val Leu Lys Pro Asn Asn Phe Ile Asp Arg Arg Lys Asp Ser 1090 1095 1100		
	Thr Leu Ser Ile Asn Asn Ile Arg Ser Thr Ile Leu Leu Ala Asn Arg 1105 1110 1115 1120		
45	Leu Tyr Ser Gly Ile Lys Val Lys Ile Gln Arg Val Asn Asn Ser Ser 1125 1130 1135		
	Thr Asn Asp Asn Leu Val Arg Lys Asn Asp Gln Val Tyr Ile Asn Phe 1140 1145 1150		
50	Val Ala Ser Lys Thr His Leu Phe Pro Leu Tyr Ala Asp Thr Ala Thr 1155 1160 1165		
	Thr Asn Lys Glu Lys Thr Ile Lys Ile Ser Ser Ser Gly Asn Arg Phe 1170 1175 1180		
	Asn Gln Val Val Val Met Asn Ser Val Gly Asn Asn Cys Thr Met Asn 1185 1190 1195 1200		
60	Phe Lys Asn Asn Asn Gly Asn Asn Ile Gly Leu Leu Gly Phe Lys Ala 1205 1210 1215		
	Asp Thr Val Val Ala Ser Thr Trp Tyr Tyr Thr His Met Arg Asp His 1220 1225 1230		
65	Thr Asn Ser Asn Gly Cys Phe Trp Asn Phe Ile Ser Glu Glu His Gly 1235 1240 1245		
70	Trp Gln Glu Lys 1250		

## (2) INFORMATION FOR SEQ ID NO:53:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1463 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "DNA"

## (ix) FEATURE:

- (A) NAME/KEY: CDS  
 (B) LOCATION: 108..1460

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:

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AGATCTCGAT CCCGCGAAAT TAATACGACT CACTATAGGG GAATTGTGAG CGGATAACAA      60
20 TTCCCCTCTA GAAATAATTT TGTTTAACTT TAAGAAGGAG ATATAACC ATG GGC CAT      116
                                     Met Gly His
                                     1
CAT CAT CAT CAT CAT CAT CAT CAT CAC AGC AGC GGC CAT ATC GAA GGT      164
His His His His His His His His His Ser Ser Gly His Ile Glu Gly
5                                     10                                     15
CGT CAT ATG GCT AGC ATG GCT CTT TCT TCT TAT ACA GAT GAT AAA ATT      212
Arg His Met Ala Ser Met Ala Leu Ser Ser Tyr Thr Asp Asp Lys Ile
20                                     25                                     30                                     35
TTA ATT TCA TAT TTT AAT AAG TTC TTT AAG AGA ATT AAA AGT AGT TCT      260
Leu Ile Ser Tyr Phe Asn Lys Phe Phe Lys Arg Ile Lys Ser Ser Ser
35                                     40                                     45                                     50
GTT TTA AAT ATG AGA TAT AAA AAT GAT AAA TAC GTA GAT ACT TCA GGA      308
Val Leu Asn Met Arg Tyr Lys Asn Asp Lys Tyr Val Asp Thr Ser Gly
55                                     60                                     65
TAT GAT TCA AAT ATA AAT ATT AAT GGA GAT GTA TAT AAA TAT CCA ACT      356
Tyr Asp Ser Asn Ile Asn Ile Asn Gly Asp Val Tyr Lys Tyr Pro Thr
70                                     75                                     80
AAT AAA AAT CAA TTT GGA ATA TAT AAT GAT AAA CTT AGT GAA GTT AAT      404
Asn Lys Asn Asn Gln Phe Gly Ile Tyr Asn Asp Lys Leu Ser Glu Val Asn
85                                     90                                     95
ATA TCT CAA AAT GAT TAC ATT ATA TAT GAT AAT AAA TAT AAA AAT TTT      452
Ile Ser Gln Asn Asp Tyr Ile Ile Tyr Asp Asn Lys Tyr Lys Asn Phe
100                                    105                                    110                                    115
AGT ATT AGT TTT TGG GTA AGA ATT CCT AAC TAT GAT AAT AAG ATA GTA      500
Ser Ile Ser Phe Trp Val Arg Ile Pro Asn Tyr Asp Asn Lys Ile Val
120                                    125                                    130
AAT GTT AAT AAT GAA TAC ACT ATA ATA AAT TGT ATG AGG GAT AAT AAT      548
Asn Val Asn Asn Glu Tyr Thr Ile Ile Asn Cys Met Arg Asp Asn Asn
135                                    140                                    145
TCA GGA TGG AAA GTA TCT CTT AAT CAT AAT GAA ATA ATT TGG ACA TTG      596
Ser Gly Trp Lys Val Ser Leu Asn His Asn Glu Ile Ile Trp Thr Leu
150                                    155                                    160
CAA GAT AAT TCA GGA ATT AAT CAA AAA TTA GCA TTT AAC TAT GGT AAC      644
Gln Asp Asn Ser Gly Ile Asn Gln Lys Leu Ala Phe Asn Tyr Gly Asn
165                                    170                                    175
GCA AAT GGT ATT TCT GAT TAT ATA AAT AAG TGG ATT TTT GTA ACT ATA      692
Ala Asn Gly Ile Ser Asp Tyr Ile Asn Lys Trp Ile Phe Val Thr Ile
180                                    185                                    190                                    195

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	ACT AAT GAT AGA TTA GGA GAT TCT AAA CTT TAT ATT AAT GGA AAT TTA Thr Asn Asp Arg Leu Gly Asp Ser Lys Leu Tyr Ile Asn Gly Asn Leu 200 205 210	740
5	ATA GAT AAA AAA TCA ATT TTA AAT TTA GGT AAT ATT CAT GTT AGT GAC Ile Asp Lys Lys Ser Ile Leu Asn Leu Gly Asn Ile His Val Ser Asp 215 220 225	788
10	AAT ATA TTA TTT AAA ATA GTT AAT TGT AGT TAT ACA AGA TAT ATT GGT Asn Ile Leu Phe Lys Ile Val Asn Cys Ser Tyr Thr Arg Tyr Ile Gly 230 235 240	836
15	ATT AGA TAT TTT AAT ATT TTT GAT AAA GAA TTA GAT GAA ACA GAA ATT Ile Arg Tyr Phe Asn Ile Phe Asp Lys Glu Leu Asp Glu Thr Glu Ile 245 250 255	884
20	CAA ACT TTA TAT AAC AAT GAA CCT AAT GCA AAT ATT TTA AAG GAT TTT Gln Thr Leu Tyr Asn Asn Glu Pro Asn Ala Asn Ile Leu Lys Asp Phe 260 265 270 275	932
	TGG GGA AAT TAT TTG CTT TAT GAC AAA GAA TAC TAT TTA TTA AAT GTG Trp Gly Asn Tyr Leu Leu Tyr Asp Lys Glu Tyr Tyr Leu Leu Asn Val 280 285 290	980
25	TTA AAA CCA AAT AAC TTT ATT AAT AGG AGA ACA GAT TCT ACT TTA AGC Leu Lys Pro Asn Asn Phe Ile Asn Arg Arg Thr Asp Ser Thr Leu Ser 295 300 305	1028
30	ATT AAT AAT ATA AGA AGC ACT ATT CTT TTA GCT AAT AGA TTA TAT AGT Ile Asn Asn Ile Arg Ser Thr Ile Leu Leu Ala Asn Arg Leu Tyr Ser 310 315 320	1076
35	GGA ATA AAA GTT AAA ATA CAA AGA GTT AAT AAT AGT AGT ACT AAC GAT Gly Ile Lys Val Lys Ile Gln Arg Val Asn Asn Ser Ser Thr Asn Asp 325 330 335	1124
40	AAT CTT GTT AGA AAG AAT GAT CAG GTA TAT ATT AAT TTT GTA GCC AGC Asn Leu Val Arg Lys Asn Asp Gln Val Tyr Ile Asn Phe Val Ala Ser 340 345 350 355	1172
	AAA ACT CAC TTA CTT CCA TTA TAT GCT GAT ACA GCT ACC ACA AAT AAA Lys Thr His Leu Leu Pro Leu Tyr Ala Asp Thr Ala Thr Thr Asn Lys 360 365 370	1220
45	GAG AAA ACA ATA AAA ATA TCA TCA TCT GGC AAT AGA TTT AAT CAA GTA Glu Lys Thr Ile Lys Ile Ser Ser Ser Gly Asn Arg Phe Asn Gln Val 375 380 385	1268
50	GTA GTT ATG AAT TCA GTA GGA AAT TGT ACA ATG AAT TTT AAA AAT AAT Val Val Met Asn Ser Val Gly Asn Cys Thr Met Asn Phe Lys Asn Asn 390 395 400	1316
55	AAT GGA AAT AAT ATT GGG TTG TTA GGT TTC AAG GCA GAT ACT GTA GTT Asn Gly Asn Asn Ile Gly Leu Leu Gly Phe Lys Ala Asp Thr Val Val 405 410 415	1364
60	GCT AGT ACT TGG TAT TAT ACA CAT ATG AGA GAT AAT ACA AAC AGC AAT Ala Ser Thr Trp Tyr Tyr Thr His Met Arg Asp Asn Thr Asn Ser Asn 420 425 430 435	1412
	GGA TTT TTT TGG AAC TTT ATT TCT GAA GAA CAT GGA TGG CAA GAA AAA Gly Phe Phe Trp Asn Phe Ile Ser Glu Glu His Gly Trp Gln Glu Lys 440 445 450	1460
65	TAA	1463

(2) INFORMATION FOR SEQ ID NO:54:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 451 amino acids

(B) TYPE: amino acid  
(D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

Met Gly His His His His His His His His His Ser Ser Gly His  
1 5 10 15  
Ile Glu Gly Arg His Met Ala Ser Met Ala Leu Ser Ser Tyr Thr Asp  
20 25 30  
Asp Lys Ile Leu Ile Ser Tyr Phe Asn Lys Phe Phe Lys Arg Ile Lys  
35 40 45  
Ser Ser Ser Val Leu Asn Met Arg Tyr Lys Asn Asp Lys Tyr Val Asp  
50 55 60  
Thr Ser Gly Tyr Asp Ser Asn Ile Asn Ile Asn Gly Asp Val Tyr Lys  
65 70 75 80  
Tyr Pro Thr Asn Lys Asn Gln Phe Gly Ile Tyr Asn Asp Lys Leu Ser  
85 90 95  
Glu Val Asn Ile Ser Gln Asn Asp Tyr Ile Ile Tyr Asp Asn Lys Tyr  
100 105 110  
Lys Asn Phe Ser Ile Ser Phe Trp Val Arg Ile Pro Asn Tyr Asp Asn  
115 120 125  
Lys Ile Val Asn Val Asn Asn Glu Tyr Thr Ile Ile Asn Cys Met Arg  
130 135 140  
Asp Asn Asn Ser Gly Trp Lys Val Ser Leu Asn His Asn Glu Ile Ile  
145 150 155 160  
Trp Thr Leu Gln Asp Asn Ser Gly Ile Asn Gln Lys Leu Ala Phe Asn  
165 170 175  
Tyr Gly Asn Ala Asn Gly Ile Ser Asp Tyr Ile Asn Lys Trp Ile Phe  
180 185 190  
Val Thr Ile Thr Asn Asp Arg Leu Gly Asp Ser Lys Leu Tyr Ile Asn  
195 200 205  
Gly Asn Leu Ile Asp Lys Lys Ser Ile Leu Asn Leu Gly Asn Ile His  
210 215 220  
Val Ser Asp Asn Ile Leu Phe Lys Ile Val Asn Cys Ser Tyr Thr Arg  
225 230 235 240  
Tyr Ile Gly Ile Arg Tyr Phe Asn Ile Phe Asp Lys Glu Leu Asp Glu  
245 250 255  
Thr Glu Ile Gln Thr Leu Tyr Asn Asn Glu Pro Asn Ala Asn Ile Leu  
260 265 270  
Lys Asp Phe Trp Gly Asn Tyr Leu Leu Tyr Asp Lys Glu Tyr Tyr Leu  
275 280 285  
Leu Asn Val Leu Lys Pro Asn Asn Phe Ile Asn Arg Arg Thr Asp Ser  
290 295 300  
Thr Leu Ser Ile Asn Asn Ile Arg Ser Thr Ile Leu Leu Ala Asn Arg  
305 310 315 320  
Leu Tyr Ser Gly Ile Lys Val Lys Ile Gln Arg Val Asn Asn Ser Ser  
325 330 335

Thr Asn Asp Asn Leu Val Arg Lys Asn Asp Gln Val Tyr Ile Asn Phe  
 340 345 350  
 5 Val Ala Ser Lys Thr His Leu Leu Pro Leu Tyr Ala Asp Thr Ala Thr  
 355 360 365  
 Thr Asn Lys Glu Lys Thr Ile Lys Ile Ser Ser Ser Gly Asn Arg Phe  
 370 375 380  
 10 Asn Gln Val Val Val Met Asn Ser Val Gly Asn Cys Thr Met Asn Phe  
 385 390 395 400  
 Lys Asn Asn Asn Gly Asn Asn Ile Gly Leu Leu Gly Phe Lys Ala Asp  
 405 410 415  
 15 Thr Val Val Ala Ser Thr Trp Tyr Tyr Thr His Met Arg Asp Asn Thr  
 420 425 430  
 20 Asn Ser Asn Gly Phe Phe Trp Asn Phe Ile Ser Glu Glu His Gly Trp  
 435 440 445  
 Gln Glu Lys  
 450  
 25 (2) INFORMATION FOR SEQ ID NO:55:  
 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 1472 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear  
 30  
 (ii) MOLECULE TYPE: other nucleic acid  
 (A) DESCRIPTION: /desc = "DNA"  
 35  
 (ix) FEATURE:  
 (A) NAME/KEY: CDS  
 (B) LOCATION: 108..1463  
 40  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:55:  
 AGATCTCGAT CCCGCGAAAT TAATACGACT CACTATAGGG GAATTGTGAG CGGATAACAA 60  
 45 TTCCCCTCTA GAAATAATTT TGTTTAACTT TAAGAAGGAG ATATACC ATG GGC CAT 116  
 Met Gly His  
 1  
 CAT CAT CAT CAT CAT CAT CAT CAC AGC AGC GGC CAT ATC GAA GGT 164  
 50 His His His His His His His His His Ser Ser Gly His Ile Glu Gly  
 5 10 15  
 CGT CAT ATG GCT AGC ATG GCT CTT TCT TCT TAT ACA GAT GAT AAA ATT 212  
 55 Arg His Met Ala Ser Met Ala Leu Ser Ser Tyr Thr Asp Asp Lys Ile  
 20 25 30 35  
 TTA ATT TCA TAT TTT AAT AAA TTC TTT AAG AGA ATT AAA AGT AGT TCA 260  
 Leu Ile Ser Tyr Phe Asn Lys Phe Phe Lys Arg Ile Lys Ser Ser Ser  
 40 45 50  
 60 GTT TTA AAT ATG AGA TAT AAA AAT GAT AAA TAC GTA GAT ACT TCA GGA 308  
 Val Leu Asn Met Arg Tyr Lys Asn Asp Lys Tyr Val Asp Thr Ser Gly  
 55 60 65  
 TAT GAT TCA AAT ATA AAT ATT AAT GGA GAT GTA TAT AAA TAT CCA ACT 356  
 65 Tyr Asp Ser Asn Ile Asn Ile Asn Gly Asp Val Tyr Lys Tyr Pro Thr  
 70 75 80  
 AAT AAA AAT CAA TTT GGA ATA TAT AAT GAT AAA CTT AGT GAA GTT AAT 404  
 70 Asn Lys Asn Gln Phe Gly Ile Tyr Asn Asp Lys Leu Ser Glu Val Asn  
 85 90 95

	ATA TCT CAA AAT GAT TAC ATT ATA TAT GAT AAT AAA TAT AAA AAT TTT Ile Ser Gln Asn Asp Tyr Ile Ile Tyr Asp Asn Lys Tyr Lys Asn Phe 100 105 110 115	452
5	AGT ATT AGT TTT TGG GTA AGA ATT CCT AAC TAT GAT AAT AAG ATA GTA Ser Ile Ser Phe Trp Val Arg Ile Pro Asn Tyr Asp Asn Lys Ile Val 120 125 130	500
10	AAT GTT AAT AAT GAA TAC ACT ATA ATA AAT TGT ATG AGA GAT AAT AAT Asn Val Asn Asn Glu Tyr Thr Ile Ile Asn Cys Met Arg Asp Asn Asn 135 140 145	548
15	TCA GGA TGG AAA GTA TCT CTT AAT CAT AAT GAA ATA ATT TGG ACA TTG Ser Gly Trp Lys Val Ser Leu Asn His Asn Glu Ile Ile Trp Thr Leu 150 155 160	596
20	CAA GAT AAT GCA GGA ATT AAT CAA AAA TTA GCA TTT AAC TAT GGT AAC Gln Asp Asn Ala Gly Ile Asn Gln Lys Leu Ala Phe Asn Tyr Gly Asn 165 170 175	644
	GCA AAT GGT ATT TCT GAT TAT ATA AAT AAG TGG ATT TTT GTA ACT ATA Ala Asn Gly Ile Ser Asp Tyr Ile Asn Lys Trp Ile Phe Val Thr Ile 180 185 190 195	692
25	ACT AAT GAT AGA TTA GGA GAT TCT AAA CTT TAT ATT AAT GGA AAT TTA Thr Asn Asp Arg Leu Gly Asp Ser Lys Leu Tyr Ile Asn Gly Asn Leu 200 205 210	740
30	ATA GAT CAA AAA TCA ATT TTA AAT TTA GGT AAT ATT CAT GTT AGT GAC Ile Asp Gln Lys Ser Ile Leu Asn Leu Gly Asn Ile His Val Ser Asp 215 220 225	788
35	AAT ATA TTA TTT AAA ATA GTT AAT TGT AGT TAT ACA AGA TAT ATT GGT Asn Ile Leu Phe Lys Ile Val Asn Cys Ser Tyr Thr Arg Tyr Ile Gly 230 235 240	836
40	ATT AGA TAT TTT AAT ATT TTT GAT AAA GAA TTA GAT GAA ACA GAA ATT Ile Arg Tyr Phe Asn Ile Phe Asp Lys Glu Leu Asp Glu Thr Glu Ile 245 250 255	884
	CAA ACT TTA TAT AGC AAT GAA CCT AAT ACA AAT ATT TTG AAG GAT TTT Gln Thr Leu Tyr Ser Asn Glu Pro Asn Thr Asn Ile Leu Lys Asp Phe 260 265 270 275	932
45	TGG GGA AAT TAT TTG CTT TAT GAC AAA GAA TAC TAT TTA TTA AAT GTG Trp Gly Asn Tyr Leu Leu Tyr Asp Lys Glu Tyr Tyr Leu Leu Asn Val 280 285 290	980
50	TTA AAA CCA AAT AAC TTT ATT GAT AGG AGA AAA GAT TCT ACT TTA AGC Leu Lys Pro Asn Asn Phe Ile Asp Arg Arg Lys Asp Ser Thr Leu Ser 295 300 305	1028
55	ATT AAT AAT ATA AGA AGC ACT ATT CTT TTA GCT AAT AGA TTA TAT AGT Ile Asn Asn Ile Arg Ser Thr Ile Leu Leu Ala Asn Arg Leu Tyr Ser 310 315 320	1076
60	GGA ATA AAA GTT AAA ATA CAA AGA GTT AAT AAT AGT AGT ACT AAC GAT Gly Ile Lys Val Lys Ile Gln Arg Val Asn Asn Ser Ser Thr Asn Asp 325 330 335	1124
	AAT CTT GTT AGA AAG AAT GAT CAG GTA TAT ATT AAT TTT GTA GCC AGC Asn Leu Val Arg Lys Asn Asp Gln Val Tyr Ile Asn Phe Val Ala Ser 340 345 350 355	1172
65	AAA ACT CAC TTA TTT CCA TTA TAT GCT GAT ACA GCT ACC ACA AAT AAA Lys Thr His Leu Phe Pro Leu Tyr Ala Asp Thr Ala Thr Thr Asn Lys 360 365 370	1220
70	GAG AAA ACA ATA AAA ATA TCA TCA TCT GGC AAT AGA TTT AAT CAA GTA Glu Lys Thr Ile Lys Ile Ser Ser Gly Asn Arg Phe Asn Gln Val	1268

	375	380	385	
5	GTA GTT ATG AAT TCA GTA GGA AAT AAT TGT ACA ATG AAT TTT AAA AAT Val Val Met Asn Ser Val Gly Asn Asn Cys Thr Met Asn Phe Lys Asn 390 395 400	1316		
10	AAT AAT GGA AAT AAT ATT GGG TTG TTA GGT TTC AAG GCA GAT ACT GTA Asn Asn Gly Asn Asn Ile Gly Leu Leu Gly Phe Lys Ala Asp Thr Val 405 410 415	1364		
15	GTT GCT AGT ACT TGG TAT TAT ACA CAT ATG AGA GAT CAT ACA AAC AGC Val Ala Ser Thr Trp Tyr Tyr Thr His Met Arg Asp His Thr Asn Ser 420 425 430 435	1412		
20	AAT GGA TGT TTT TGG AAC TTT ATT TCT GAA GAA CAT GGA TGG CAA GAA Asn Gly Cys Phe Trp Asn Phe Ile Ser Glu Glu His Gly Trp Gln Glu 440 445 450	1460		
25	AAA TAAAAGCTT Lys	1472		
(2) INFORMATION FOR SEQ ID NO:56:				
(i) SEQUENCE CHARACTERISTICS:				
(A) LENGTH: 452 amino acids				
(B) TYPE: amino acid				
(D) TOPOLOGY: linear				
(ii) MOLECULE TYPE: protein				
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:56:				
35	Met Gly His His His His His His His His His His Ser Ser Gly His 1 5 10 15			
40	Ile Glu Gly Arg His Met Ala Ser Met Ala Leu Ser Ser Tyr Thr Asp 20 25 30			
45	Asp Lys Ile Leu Ile Ser Tyr Phe Asn Lys Phe Phe Lys Arg Ile Lys 35 40 45			
50	Ser Ser Ser Val Leu Asn Met Arg Tyr Lys Asn Asp Lys Tyr Val Asp 50 55 60			
55	Thr Ser Gly Tyr Asp Ser Asn Ile Asn Ile Asn Gly Asp Val Tyr Lys 65 70 75 80			
60	Tyr Pro Thr Asn Lys Asn Gln Phe Gly Ile Tyr Asn Asp Lys Leu Ser 85 90 95			
65	Glu Val Asn Ile Ser Gln Asn Asp Tyr Ile Ile Tyr Asp Asn Lys Tyr 100 105 110			
70	Lys Asn Phe Ser Ile Ser Phe Trp Val Arg Ile Pro Asn Tyr Asp Asn 115 120 125			
	Lys Ile Val Asn Val Asn Asn Glu Tyr Thr Ile Ile Asn Cys Met Arg 130 135 140			
	Asp Asn Asn Ser Gly Trp Lys Val Ser Leu Asn His Asn Glu Ile Ile 145 150 155 160			
	Trp Thr Leu Gln Asp Asn Ala Gly Ile Asn Gln Lys Leu Ala Phe Asn 165 170 175			
	Tyr Gly Asn Ala Asn Gly Ile Ser Asp Tyr Ile Asn Lys Trp Ile Phe 180 185 190			
	Val Thr Ile Thr Asn Asp Arg Leu Gly Asp Ser Lys Leu Tyr Ile Asn 195 200 205			

Gly Asn Leu Ile Asp Gln Lys Ser Ile Leu Asn Leu Gly Asn Ile His  
 210 215 220  
 5 Val Ser Asp Asn Ile Leu Phe Lys Ile Val Asn Cys Ser Tyr Thr Arg  
 225 230 235 240  
 Tyr Ile Gly Ile Arg Tyr Phe Asn Ile Phe Asp Lys Glu Leu Asp Glu  
 245 250 255  
 10 Thr Glu Ile Gln Thr Leu Tyr Ser Asn Glu Pro Asn Thr Asn Ile Leu  
 260 265 270  
 Lys Asp Phe Trp Gly Asn Tyr Leu Leu Tyr Asp Lys Glu Tyr Tyr Leu  
 275 280 285  
 15 Leu Asn Val Leu Lys Pro Asn Asn Phe Ile Asp Arg Arg Lys Asp Ser  
 290 295 300  
 20 Thr Leu Ser Ile Asn Asn Ile Arg Ser Thr Ile Leu Leu Ala Asn Arg  
 305 310 315 320  
 Leu Tyr Ser Gly Ile Lys Val Lys Ile Gln Arg Val Asn Asn Ser Ser  
 325 330 335  
 25 Thr Asn Asp Asn Leu Val Arg Lys Asn Asp Gln Val Tyr Ile Asn Phe  
 340 345 350  
 Val Ala Ser Lys Thr His Leu Phe Pro Leu Tyr Ala Asp Thr Ala Thr  
 355 360 365  
 30 Thr Asn Lys Glu Lys Thr Ile Lys Ile Ser Ser Ser Gly Asn Arg Phe  
 370 375 380  
 35 Asn Gln Val Val Val Met Asn Ser Val Gly Asn Asn Cys Thr Met Asn  
 385 390 395 400  
 Phe Lys Asn Asn Asn Gly Asn Asn Ile Gly Leu Leu Gly Phe Lys Ala  
 405 410 415  
 40 Asp Thr Val Val Ala Ser Thr Trp Tyr Tyr Thr His Met Arg Asp His  
 420 425 430  
 Thr Asn Ser Asn Gly Cys Phe Trp Asn Phe Ile Ser Glu Glu His Gly  
 435 440 445  
 45 Trp Gln Glu Lys  
 450

50 (2) INFORMATION FOR SEQ ID NO:57:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 31 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

55

- (ii) MOLECULE TYPE: other nucleic acid
- (A) DESCRIPTION: /desc = "DNA"

60

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:57:

CGCCATGGCT CTTTCTTCTT ATACAGATGA T

31

65

(2) INFORMATION FOR SEQ ID NO:58:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 29 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

70



(ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "DNA"

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:58:

GCAAGCTTTT ATTTTCTTG CCATCCATG

29

(2) INFORMATION FOR SEQ ID NO:59:

10 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 3876 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: double  
15 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

20 (A) NAME/KEY: CDS  
(B) LOCATION: 1..3873

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:59:

25	ATG CCA ATA ACA ATT AAC AAC TTT AAT TAT TCA GAT CCT GTT GAT AAT Met Pro Ile Thr Ile Asn Asn Phe Asn Tyr Ser Asp Pro Val Asp Asn 1 5 10 15	48
30	AAA AAT ATT TTA TAT TTA GAT ACT CAT TTA AAT ACA CTA GCT AAT GAG Lys Asn Ile Leu Tyr Leu Asp Thr His Leu Asn Thr Leu Ala Asn Glu 20 25 30	96
35	CCT GAA AAA GCC TTT CGC ATT ACA GGA AAT ATA TGG GTA ATA CCT GAT Pro Glu Lys Ala Phe Arg Ile Thr Gly Asn Ile Trp Val Ile Pro Asp 35 40 45	144
40	AGA TTT TCA AGA AAT TCT AAT CCA AAT TTA AAT AAA CCT CCT CGA GTT Arg Phe Ser Arg Asn Ser Asn Pro Asn Leu Asn Lys Pro Pro Arg Val 50 55 60	192
45	ACA AGC CCT AAA AGT GGT TAT TAT GAT CCT AAT TAT TTG AGT ACT GAT Thr Ser Pro Lys Ser Gly Tyr Tyr Asp Pro Asn Tyr Leu Ser Thr Asp 65 70 75 80	240
50	TCT GAC AAA GAT ACA TTT TTA AAA GAA ATT ATA AAG TTA TTT AAA AGA Ser Asp Lys Asp Thr Phe Leu Lys Glu Ile Ile Lys Leu Phe Lys Arg 85 90 95	288
55	ATT AAT TCT AGA GAA ATA GGA GAA GAA TTA ATA TAT AGA CTT TCG ACA Ile Asn Ser Arg Glu Ile Gly Glu Glu Leu Ile Tyr Arg Leu Ser Thr 100 105 110	336
60	GAT ATA CCC TTT CCT GGG AAT AAC AAT ACT CCA ATT AAT ACT TTT GAT Asp Ile Pro Phe Pro Gly Asn Asn Asn Thr Pro Ile Asn Thr Phe Asp 115 120 125	384
65	TTT GAT GTA GAT TTT AAC AGT GTT GAT GTT AAA ACT AGA CAA GGT AAC Phe Asp Val Asp Phe Asn Ser Val Asp Val Lys Thr Arg Gln Gly Asn 130 135 140	432
70	AAC TGG GTT AAA ACT GGT AGC ATA AAT CCT AGT GTT ATA ATA ACT GGA Asn Trp Val Lys Thr Gly Ser Ile Asn Pro Ser Val Ile Ile Thr Gly 145 150 155 160	480
75	CCT AGA GAA AAC ATT ATA GAT CCA GAA ACT TCT ACG TTT AAA TTA ACT Pro Arg Glu Asn Ile Ile Asp Pro Glu Thr Ser Thr Phe Lys Leu Thr 165 170 175	528

	AAC AAT ACT TTT GCG GCA CAA GAA GGA TTT GGT GCT TTA TCA ATA ATT	576
	Asn Asn Thr Phe Ala Ala Gln Glu Gly Phe Gly Ala Leu Ser Ile Ile	
	180 185 190	
5	TCA ATA TCA CCT AGA TTT ATG CTA ACA TAT AGT AAT GCA ACT AAT GAT	624
	Ser Ile Ser Pro Arg Phe Met Leu Thr Tyr Ser Asn Ala Thr Asn Asp	
	195 200 205	
10	GTA GGA GAG GGT AGA TTT TCT AAG TCT GAA TTT TGC ATG GAT CCA ATA	672
	Val Gly Glu Gly Arg Phe Ser Lys Ser Glu Phe Cys Met Asp Pro Ile	
	210 215 220	
15	CTA ATT TTA ATG CAT GAA CTT AAT CAT GCA ATG CAT AAT TTA TAT GGA	720
	Leu Ile Leu Met His Glu Leu Asn His Ala Met His Asn Leu Tyr Gly	
	225 230 235 240	
20	ATA GCT ATA CCA AAT GAT CAA ACA ATT TCA TCT GTA ACT AGT AAT ATT	768
	Ile Ala Ile Pro Asn Asp Gln Thr Ile Ser Ser Val Thr Ser Asn Ile	
	245 250 255	
25	TTT TAT TCT CAA TAT AAT GTG AAA TTA GAG TAT GCA GAA ATA TAT GCA	816
	Phe Tyr Ser Gln Tyr Asn Val Lys Leu Glu Tyr Ala Glu Ile Tyr Ala	
	260 265 270	
30	TTT GGA GGT CCA ACT ATA GAC CTT ATT CCT AAA AGT GCA AGG AAA TAT	864
	Phe Gly Gly Pro Thr Ile Asp Leu Ile Pro Lys Ser Ala Arg Lys Tyr	
	275 280 285	
35	TTT GAG GAA AAG GCA TTG GAT TAT TAT AGA TCT ATA GCT AAA AGA CTT	912
	Phe Glu Glu Lys Ala Leu Asp Tyr Tyr Arg Ser Ile Ala Lys Arg Leu	
	290 295 300	
40	AAT AGT ATA ACT ACT GCA AAT CCT TCA AGC TTT AAT AAA TAT ATA GGG	960
	Asn Ser Ile Thr Thr Ala Asn Pro Ser Ser Phe Asn Lys Tyr Ile Gly	
	305 310 315 320	
45	GAA TAT AAA CAG AAA CTT ATT AGA AAG TAT AGA TTC GTA GTA GAA TCT	1008
	Glu Tyr Lys Gln Lys Leu Ile Arg Lys Tyr Arg Phe Val Val Glu Ser	
	325 330 335	
50	TCA GGT GAA GTT ACA GTA AAT CGT AAT AAG TTT GTT GAG TTA TAT AAT	1056
	Ser Gly Glu Val Thr Val Asn Arg Asn Lys Phe Val Glu Leu Tyr Asn	
	340 345 350	
55	GAA CTT ACA CAA ATA TTT ACA GAA TTT AAC TAC GCT AAA ATA TAT AAT	1104
	Glu Leu Thr Gln Ile Phe Thr Glu Phe Asn Tyr Ala Lys Ile Tyr Asn	
	355 360 365	
60	GTA CAA AAT AGG AAA ATA TAT CTT TCA AAT GTA TAT ACT CCG GTT ACG	1152
	Val Gln Asn Arg Lys Ile Tyr Leu Ser Asn Val Tyr Thr Pro Val Thr	
	370 375 380	
65	GCG AAT ATA TTA GAC GAT AAT GTT TAT GAT ATA CAA AAT GGA TTT AAT	1200
	Ala Asn Ile Leu Asp Asp Asn Val Tyr Asp Ile Gln Asn Gly Phe Asn	
	385 390 395 400	
70	ATA CCT AAA AGT AAT TTA AAT GTA CTA TTT ATG GGT CAA AAT TTA TCT	1248
	Ile Pro Lys Ser Asn Leu Asn Val Leu Phe Met Gly Gln Asn Leu Ser	
	405 410 415	
75	CGA AAT CCA GCA TTA AGA AAA GTC AAT CCT GAA AAT ATG CTT TAT TTA	1296
	Arg Asn Pro Ala Leu Arg Lys Val Asn Pro Glu Asn Met Leu Tyr Leu	
	420 425 430	
80	TTT ACA AAA TTT TGT CAT AAA GCA ATA GAT GGT AGA TCA TTA TAT AAT	1344
	Phe Thr Lys Phe Cys His Lys Ala Ile Asp Gly Arg Ser Leu Tyr Asn	
	435 440 445	
85	AAA ACA TTA GAT TGT AGA GAG CTT TTA GTT AAA AAT ACT GAC TTA CCC	1392
	Lys Thr Leu Asp Cys Arg Glu Leu Leu Val Lys Asn Thr Asp Leu Pro	

	450	455	460	
5	TTT ATA GGT GAT ATT AGT GAT GTT AAA ACT GAT ATA TTT TTA AGA AAA Phe Ile Gly Asp Ile Ser Asp Val Lys Thr Asp Ile Phe Leu Arg Lys 465 470 475 480	1440		
10	GAT ATT AAT GAA GAA ACT GAA GTT ATA TAC TAT CCG GAC AAT GTT TCA Asp Ile Asn Glu Thr Glu Val Ile Tyr Pro Asp Asn Val Ser 485 490 495	1488		
15	GTA GAT CAA GTT ATT CTC AGT AAG AAT ACC TCA GAA CAT GGA CAA CTA Val Asp Gln Val Ile Leu Ser Lys Asn Thr Ser Glu His Gly Gln Leu 500 505 510	1536		
20	GAT TTA TTA TAC CCT AGT ATT GAC AGT GAG AGT GAA ATA TTA CCA GGG Asp Leu Leu Tyr Pro Ser Ile Asp Ser Glu Ser Glu Ile Leu Pro Gly 515 520 525	1584		
25	GAG AAT CAA GTC TTT TAT GAT AAT AGA ACT CAA AAT GTT GAT TAT TTG Glu Asn Gln Val Phe Tyr Asp Asn Arg Thr Gln Asn Val Asp Tyr Leu 530 535 540	1632		
30	AAT TCT TAT TAT TAC CTA GAA TCT CAA AAA CTA AGT GAT AAT GTT GAA Asn Ser Tyr Tyr Tyr Leu Glu Ser Gln Lys Leu Ser Asp Asn Val Glu 545 550 555 560	1680		
35	GAT TTT ACT TTT ACG AGA TCA ATT GAG GAG GCT TTG GAT AAT AGT GCA Asp Phe Thr Phe Thr Arg Ser Ile Glu Glu Ala Leu Asp Asn Ser Ala 565 570 575	1728		
40	AAA GTA TAT ACT TAC TTT CCT ACA CTA GCT AAT AAA GTA AAT GCG GGT Lys Val Tyr Thr Tyr Phe Pro Thr Leu Ala Asn Lys Val Asn Ala Gly 580 585 590	1776		
45	GTT CAA GGT GGT TTA TTT TTA ATG TGG GCA AAT GAT GTA GTT GAA GAT Val Gln Gly Gly Leu Phe Leu Met Trp Ala Asn Asp Val Val Glu Asp 595 600 605	1824		
50	TTT ACT ACA AAT ATT CTA AGA AAA GAT ACA TTA GAT AAA ATA TCA GAT Phe Thr Thr Asn Ile Leu Arg Lys Asp Thr Leu Asp Lys Ile Ser Asp 610 615 620	1872		
55	GTA TCA GCT ATT ATT CCC TAT ATA GGA CCC GCA TTA AAT ATA AGT AAT Val Ser Ala Ile Ile Pro Tyr Ile Gly Pro Ala Leu Asn Ile Ser Asn 625 630 635 640	1920		
60	TCT GTA AGA AGA GGA AAT TTT ACT GAA GCA TTT GCA GTT ACT GGT GTA Ser Val Arg Arg Gly Asn Phe Thr Glu Ala Phe Ala Val Thr Gly Val 645 650 655	1968		
65	ACT ATT TTA TTA GAA GCA TTT CCT GAA TTT ACA ATA CCT GCA CTT GGT Thr Ile Leu Leu Glu Ala Phe Pro Glu Phe Thr Ile Pro Ala Leu Gly 660 665 670	2016		
70	GCA TTT GTG ATT TAT AGT AAG GTT CAA GAA AGA AAC GAG ATT ATT AAA Ala Phe Val Ile Tyr Ser Lys Val Gln Glu Arg Asn Glu Ile Ile Lys 675 680 685	2064		
75	ACT ATA GAT AAT TGT TTA GAA CAA AGG ATT AAG AGA TGG AAA GAT TCA Thr Ile Asp Asn Cys Leu Glu Gln Arg Ile Lys Arg Trp Lys Asp Ser 690 695 700	2112		
80	TAT GAA TGG ATG ATG GGA ACG TGG TTA TCC AGG ATT ATT ACT CAA TTT Tyr Glu Trp Met Met Gly Thr Trp Leu Ser Arg Ile Ile Thr Gln Phe 705 710 715 720	2160		
85	AAT AAT ATA AGT TAT CAA ATG TAT GAT TCT TTA AAT TAT CAG GCA GGT Asn Asn Ile Ser Tyr Gln Met Tyr Asp Ser Leu Asn Tyr Gln Ala Gly 725 730 735	2208		

	GCA ATC AAA GCT AAA ATA GAT TTA GAA TAT AAA AAA TAT TCA GGA AGT Ala Ile Lys Ala Lys Ile Asp Leu Glu Tyr Lys Lys Tyr Ser Gly Ser 740 745 750	2256
5	GAT AAA GAA AAT ATA AAA AGT CAA GTT GAA AAT TTA AAA AAT AGT TTA Asp Lys Glu Asn Ile Lys Ser Gln Val Glu Asn Leu Lys Asn Ser Leu 755 760 765	2304
10	GAT GTA AAA ATT TCG GAA GCA ATG AAT AAT ATA AAT AAA TTT ATA CGA Asp Val Lys Ile Ser Glu Ala Met Asn Asn Ile Asn Lys Phe Ile Arg 770 775 780	2352
15	GAA TGT TCC GTA ACA TAT TTA TTT AAA AAT ATG TTA CCT AAA GTA ATT Glu Cys Ser Val Thr Tyr Leu Phe Lys Asn Met Leu Pro Lys Val Ile 785 790 795 800	2400
20	GAT GAA TTA AAT GAG TTT GAT CGA AAT ACT AAA GCA AAA TTA ATT AAT Asp Glu Leu Asn Glu Phe Asp Arg Asn Thr Lys Ala Lys Leu Ile Asn 805 810 815	2448
	CTT ATA GAT AGT CAT AAT ATT ATT CTA GTT GGT GAA GTA GAT AAA TTA Leu Ile Asp Ser His Asn Ile Ile Leu Val Gly Glu Val Asp Lys Leu 820 825 830	2496
25	AAA GCA AAA GTA AAT AAT AGC TTT CAA AAT ACA ATA CCC TTT AAT ATT Lys Ala Lys Val Asn Asn Ser Phe Gln Asn Thr Ile Pro Phe Asn Ile 835 840 845	2544
30	TTT TCA TAT ACT AAT AAT TCT TTA TTA AAA GAT ATA ATT AAT GAA TAT Phe Ser Tyr Thr Asn Asn Ser Leu Leu Lys Asp Ile Ile Asn Glu Tyr 850 855 860	2592
35	TTC AAT AAT ATT AAT GAT TCA AAA ATT TTG AGC CTA CAA AAC AGA AAA Phe Asn Asn Ile Asn Asp Ser Lys Ile Leu Ser Leu Gln Asn Arg Lys 865 870 875 880	2640
40	AAT ACT TTA GTG GAT ACA TCA GGA TAT AAT GCA GAA GTG AGT GAA GAA Asn Thr Leu Val Asp Thr Ser Gly Tyr Asn Ala Glu Val Ser Glu Glu 885 890 895	2688
	GGC GAT GTT CAG CTT AAT CCA ATA TTT CCA TTT GAC TTT AAA TTA GGT Gly Asp Val Gln Leu Asn Pro Ile Phe Pro Phe Asp Phe Lys Leu Gly 900 905 910	2736
45	AGT TCA GGG GAG GAT AGA GGT AAA GTT ATA GTA ACC CAG AAT GAA AAT Ser Ser Gly Glu Asp Arg Gly Lys Val Ile Val Thr Gln Asn Glu Asn 915 920 925	2784
50	ATT GTA TAT AAT TCT ATG TAT GAA AGT TTT AGC ATT AGT TTT TGG ATT Ile Val Tyr Asn Ser Met Tyr Glu Ser Phe Ser Ile Ser Phe Trp Ile 930 935 940	2832
55	AGA ATA AAT AAA TGG GTA AGT AAT TTA CCT GGA TAT ACT ATA ATT GAT Arg Ile Asn Lys Trp Val Ser Asn Leu Pro Gly Tyr Thr Ile Ile Asp 945 950 955 960	2880
60	AGT GTT AAA AAT AAC TCA GGT TGG AGT ATA GGT ATT ATT AGT AAT TTT Ser Val Lys Asn Asn Ser Gly Trp Ser Ile Gly Ile Ile Ser Asn Phe 965 970 975	2928
	TTA GTA TTT ACT TTA AAA CAA AAT GAA GAT AGT GAA CAA AGT ATA AAT Leu Val Phe Thr Leu Lys Gln Asn Glu Asp Ser Glu Gln Ser Ile Asn 980 985 990	2976
65	TTT AGT TAT GAT ATA TCA AAT AAT CCT CCT GGA TAC AAT AAA TGG TTT Phe Ser Tyr Asp Ile Ser Asn Asn Ala Pro Gly Tyr Asn Lys Trp Phe 995 1000 1005	3024
70	TTT GTA ACT GTT ACT AAC AAT ATG ATG GGA AAT ATG AAG ATT TAT ATA Phe Val Thr Val Thr Asn Asn Met Met Gly Asn Met Lys Ile Tyr Ile	3072

	1010	1015	1020	
5	AAT GGA AAA TTA ATA GAT ACT ATA AAA GTT AAA GAA CTA ACT GGA ATT Asn Gly Lys Leu Ile Asp Thr Ile Lys Val Lys Glu Leu Thr Gly Ile 1025 1030 1035 1040			3120
10	AAT TTT AGC AAA ACT ATA ACA TTT GAA ATA AAT AAA ATT CCA GAT ACC Asn Phe Ser Lys Thr Ile Thr Phe Glu Ile Asn Lys Ile Pro Asp Thr 1045 1050 1055			3168
15	GGT TTG ATT ACT TCA GAT TCT GAT AAC ATC AAT ATG TGG ATA AGA GAT Gly Leu Ile Thr Ser Asp Ser Asp Asn Ile Asn Met Trp Ile Arg Asp 1060 1065 1070			3216
20	TTT TAT ATA TTT GCT AAA GAA TTA GAT GGT AAA GAT ATT AAT ATA TTA Phe Tyr Ile Phe Ala Lys Glu Leu Asp Gly Lys Asp Ile Asn Ile Leu 1075 1080 1085			3264
25	TTT AAT AGC TTG CAA TAT ACT AAT GTT GTA AAA GAT TAT TGG GGA AAT Phe Asn Ser Leu Gln Tyr Thr Asn Val Val Lys Asp Tyr Trp Gly Asn 1090 1095 1100			3312
30	GAT TTA AGA TAT AAT AAA GAA TAT TAT ATG GTT AAT ATA GAT TAT TTA Asp Leu Arg Tyr Asn Lys Glu Tyr Tyr Met Val Asn Ile Asp Tyr Leu 1105 1110 1115 1120			3360
35	AAT AGA TAT ATG TAT GCG AAC TCA CGA CAA ATT GTT TTT AAT ACA CGT Asn Arg Tyr Met Tyr Ala Asn Ser Arg Gln Ile Val Phe Asn Thr Arg 1125 1130 1135			3408
40	AGA AAT AAT AAT GAC TTC AAT GAA GGA TAT AAA ATT ATA ATA AAA AGA Arg Asn Asn Asn Asp Phe Asn Glu Gly Tyr Lys Ile Ile Ile Lys Arg 1140 1145 1150			3456
45	ATC AGA GGA AAT ACA AAT GAT ACT AGA GTA CGA GGA GGA GAT ATT TTA Ile Arg Gly Asn Thr Asn Asp Thr Arg Val Arg Gly Gly Asp Ile Leu 1155 1160 1165			3504
50	TAT TTT GAT ATG ACA ATT AAT AAC AAA GCA TAT AAT TTG TTT ATG AAG Tyr Phe Asp Met Thr Ile Asn Asn Lys Ala Tyr Asn Leu Phe Met Lys 1170 1175 1180			3552
55	AAT GAA ACT ATG TAT GCA GAT AAT CAT AGT ACT GAA GAT ATA TAT GCT Asn Glu Thr Met Tyr Ala Asp Asn His Ser Thr Glu Asp Ile Tyr Ala 1185 1190 1195 1200			3600
60	ATA GGT TTA AGA GAA CAA ACA AAG GAT ATA AAT GAT AAT ATT ATA TTT Ile Gly Leu Arg Glu Gln Thr Lys Asp Ile Asn Asp Asn Ile Ile Phe 1205 1210 1215			3648
65	CAA ATA CAA CCA ATG AAT AAT ACT TAT TAT TAC GCA TCT CAA ATA TTT Gln Ile Gln Pro Met Asn Asn Thr Tyr Tyr Tyr Ala Ser Gln Ile Phe 1220 1225 1230			3696
70	AAA TCA AAT TTT AAT GGA GAA AAT ATT TCT GGA ATA TGT TCA ATA GGT Lys Ser Asn Phe Asn Gly Glu Asn Ile Ser Gly Ile Cys Ser Ile Gly 1235 1240 1245			3744
75	ACT TAT CGT TTT AGA CTT GGA GGT GAT TGG TAT AGA CAC AAT TAT TTG Thr Tyr Arg Phe Arg Leu Gly Gly Asp Trp Tyr Arg His Asn Tyr Leu 1250 1255 1260			3792
80	GTG CCT ACT GTG AAG CAA GGA AAT TAT GCT TCA TTA TTA GAA TCA ACA Val Pro Thr Val Lys Gln Gly Asn Tyr Ala Ser Leu Leu Glu Ser Thr 1265 1270 1275 1280			3840
85	TCA ACT CAT TGG GGT TTT GTA CCT GTA AGT GAA TAA Ser Thr His Trp Gly Phe Val Pro Val Ser Glu 1285 1290			3876

## (2) INFORMATION FOR SEQ ID NO:60:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1291 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:60:

Met Pro Ile Thr Ile Asn Asn Phe Asn Tyr Ser Asp Pro Val Asp Asn  
 1 5 10 15  
 Lys Asn Ile Leu Tyr Leu Asp Thr His Leu Asn Thr Leu Ala Asn Glu  
 20 25 30  
 Pro Glu Lys Ala Phe Arg Ile Thr Gly Asn Ile Trp Val Ile Pro Asp  
 35 40 45  
 Arg Phe Ser Arg Asn Ser Asn Pro Asn Leu Asn Lys Pro Pro Arg Val  
 50 55 60  
 Thr Ser Pro Lys Ser Gly Tyr Tyr Asp Pro Asn Tyr Leu Ser Thr Asp  
 65 70 75 80  
 Ser Asp Lys Asp Thr Phe Leu Lys Glu Ile Ile Lys Leu Phe Lys Arg  
 85 90 95  
 Ile Asn Ser Arg Glu Ile Gly Glu Glu Leu Ile Tyr Arg Leu Ser Thr  
 100 105 110  
 Asp Ile Pro Phe Pro Gly Asn Asn Asn Thr Pro Ile Asn Thr Phe Asp  
 115 120 125  
 Phe Asp Val Asp Phe Asn Ser Val Asp Val Lys Thr Arg Gln Gly Asn  
 130 135 140  
 Asn Trp Val Lys Thr Gly Ser Ile Asn Pro Ser Val Ile Ile Thr Gly  
 145 150 155 160  
 Pro Arg Glu Asn Ile Ile Asp Pro Glu Thr Ser Thr Phe Lys Leu Thr  
 165 170 175  
 Asn Asn Thr Phe Ala Ala Gln Glu Gly Phe Gly Ala Leu Ser Ile Ile  
 180 185 190  
 Ser Ile Ser Pro Arg Phe Met Leu Thr Tyr Ser Asn Ala Thr Asn Asp  
 195 200 205  
 Val Gly Glu Gly Arg Phe Ser Lys Ser Glu Phe Cys Met Asp Pro Ile  
 210 215 220  
 Leu Ile Leu Met His Glu Leu Asn His Ala Met His Asn Leu Tyr Gly  
 225 230 235 240  
 Ile Ala Ile Pro Asn Asp Gln Thr Ile Ser Ser Val Thr Ser Asn Ile  
 245 250 255  
 Phe Tyr Ser Gln Tyr Asn Val Lys Leu Glu Tyr Ala Glu Ile Tyr Ala  
 260 265 270  
 Phe Gly Gly Pro Thr Ile Asp Leu Ile Pro Lys Ser Ala Arg Lys Tyr  
 275 280 285  
 Phe Glu Glu Lys Ala Leu Asp Tyr Tyr Arg Ser Ile Ala Lys Arg Leu  
 290 295 300  
 Asn Ser Ile Thr Thr Ala Asn Pro Ser Ser Phe Asn Lys Tyr Ile Gly  
 305 310 315 320

Glu Tyr Lys Gln Lys Leu Ile Arg Lys Tyr Arg Phe Val Val Glu Ser  
 325 330 335  
 5 Ser Gly Glu Val Thr Val Asn Arg Asn Lys Phe Val Glu Leu Tyr Asn  
 340 345 350  
 Glu Leu Thr Gln Ile Phe Thr Glu Phe Asn Tyr Ala Lys Ile Tyr Asn  
 355 360 365  
 10 Val Gln Asn Arg Lys Ile Tyr Leu Ser Asn Val Tyr Thr Pro Val Thr  
 370 375 380  
 Ala Asn Ile Leu Asp Asp Asn Val Tyr Asp Ile Gln Asn Gly Phe Asn  
 385 390 395 400  
 15 Ile Pro Lys Ser Asn Leu Asn Val Leu Phe Met Gly Gln Asn Leu Ser  
 405 410 415  
 20 Arg Asn Pro Ala Leu Arg Lys Val Asn Pro Glu Asn Met Leu Tyr Leu  
 420 425 430  
 Phe Thr Lys Phe Cys His Lys Ala Ile Asp Gly Arg Ser Leu Tyr Asn  
 435 440 445  
 25 Lys Thr Leu Asp Cys Arg Glu Leu Leu Val Lys Asn Thr Asp Leu Pro  
 450 455 460  
 Phe Ile Gly Asp Ile Ser Asp Val Lys Thr Asp Ile Phe Leu Arg Lys  
 465 470 475 480  
 30 Asp Ile Asn Glu Glu Thr Glu Val Ile Tyr Tyr Pro Asp Asn Val Ser  
 485 490 495  
 35 Val Asp Gln Val Ile Leu Ser Lys Asn Thr Ser Glu His Gly Gln Leu  
 500 505 510  
 Asp Leu Leu Tyr Pro Ser Ile Asp Ser Glu Ser Glu Ile Leu Pro Gly  
 515 520 525  
 40 Glu Asn Gln Val Phe Tyr Asp Asn Arg Thr Gln Asn Val Asp Tyr Leu  
 530 535 540  
 Asn Ser Tyr Tyr Tyr Leu Glu Ser Gln Lys Leu Ser Asp Asn Val Glu  
 545 550 555 560  
 45 Asp Phe Thr Phe Thr Arg Ser Ile Glu Glu Ala Leu Asp Asn Ser Ala  
 565 570 575  
 50 Lys Val Tyr Thr Tyr Phe Pro Thr Leu Ala Asn Lys Val Asn Ala Gly  
 580 585 590  
 Val Gln Gly Gly Leu Phe Leu Met Trp Ala Asn Asp Val Val Glu Asp  
 595 600 605  
 55 Phe Thr Thr Asn Ile Leu Arg Lys Asp Thr Leu Asp Lys Ile Ser Asp  
 610 615 620  
 Val Ser Ala Ile Ile Pro Tyr Ile Gly Pro Ala Leu Asn Ile Ser Asn  
 625 630 635 640  
 60 Ser Val Arg Arg Gly Asn Phe Thr Glu Ala Phe Ala Val Thr Gly Val  
 645 650 655  
 65 Thr Ile Leu Leu Glu Ala Phe Pro Glu Phe Thr Ile Pro Ala Leu Gly  
 660 665 670  
 Ala Phe Val Ile Tyr Ser Lys Val Gln Glu Arg Asn Glu Ile Ile Lys  
 675 680 685  
 70 Thr Ile Asp Asn Cys Leu Glu Gln Arg Ile Lys Arg Trp Lys Asp Ser

	690	695	700
5	Tyr 705	Glu Trp Met Met Gly Thr Trp Leu Ser Arg Ile Ile Thr Gln Phe 710 715 720	
	Asn Asn Ile Ser Tyr 725	Gln Met Tyr Asp Ser Leu Asn Tyr Gln Ala Gly 730 735	
10	Ala Ile Lys Ala Lys Ile Asp Leu Glu Tyr Lys Lys Tyr Ser Gly Ser 740 745 750		
	Asp Lys 755	Glu Asn Ile Lys Ser Gln Val Glu Asn Leu Lys Asn Ser Leu 760 765	
15	Asp Val Lys Ile Ser Glu Ala Met Asn Asn Ile Asn Lys Phe Ile Arg 770 775 780		
	Glu Cys Ser Val Thr Tyr Leu Phe Lys Asn Met Leu Pro Lys Val Ile 785 790 795 800		
20	Asp Glu Leu Asn Glu Phe Asp Arg Asn Thr Lys Ala Lys Leu Ile Asn 805 810 815		
25	Leu Ile Asp Ser His Asn Ile Ile Leu Val Gly Glu Val Asp Lys Leu 820 825 830		
	Lys Ala Lys Val Asn Asn Ser Phe Gln Asn Thr Ile Pro Phe Asn Ile 835 840 845		
30	Phe Ser Tyr Thr Asn Asn Ser Leu Leu Lys Asp Ile Ile Asn Glu Tyr 850 855 860		
	Phe Asn Asn Ile Asn Asp Ser Lys Ile Leu Ser Leu Gln Asn Arg Lys 865 870 875 880		
35	Asn Thr Leu Val Asp Thr Ser Gly Tyr Asn Ala Glu Val Ser Glu Glu 885 890 895		
40	Gly Asp Val Gln Leu Asn Pro Ile Phe Pro Phe Asp Phe Lys Leu Gly 900 905 910		
	Ser Ser Gly Glu Asp Arg Gly Lys Val Ile Val Thr Gln Asn Glu Asn 915 920 925		
45	Ile Val Tyr Asn Ser Met Tyr Glu Ser Phe Ser Ile Ser Phe Trp Ile 930 935 940		
	Arg Ile Asn Lys Trp Val Ser Asn Leu Pro Gly Tyr Thr Ile Ile Asp 945 950 955 960		
50	Ser Val Lys Asn Asn Ser Gly Trp Ser Ile Gly Ile Ile Ser Asn Phe 965 970 975		
55	Leu Val Phe Thr Leu Lys Gln Asn Glu Asp Ser Glu Gln Ser Ile Asn 980 985 990		
	Phe Ser Tyr Asp Ile Ser Asn Asn Ala Pro Gly Tyr Asn Lys Trp Phe 995 1000 1005		
60	Phe Val Thr Val Thr Asn Asn Met Met Gly Asn Met Lys Ile Tyr Ile 1010 1015 1020		
	Asn Gly Lys Leu Ile Asp Thr Ile Lys Val Lys Glu Leu Thr Gly Ile 1025 1030 1035 1040		
65	Asn Phe Ser Lys Thr Ile Thr Phe Glu Ile Asn Lys Ile Pro Asp Thr 1045 1050 1055		
70	Gly Leu Ile Thr Ser Asp Ser Asp Asn Ile Asn Met Trp Ile Arg Asp 1060 1065 1070		



Phe Tyr Ile Phe Ala Lys Glu Leu Asp Gly Lys Asp Ile Asn Ile Leu  
 1075 1080 1085  
 5 Phe Asn Ser Leu Gln Tyr Thr Asn Val Val Lys Asp Tyr Trp Gly Asn  
 1090 1095 1100  
 Asp Leu Arg Tyr Asn Lys Glu Tyr Tyr Met Val Asn Ile Asp Tyr Leu  
 1105 1110 1115 1120  
 10 Asn Arg Tyr Met Tyr Ala Asn Ser Arg Gln Ile Val Phe Asn Thr Arg  
 1125 1130 1135  
 Arg Asn Asn Asn Asp Phe Asn Glu Gly Tyr Lys Ile Ile Ile Lys Arg  
 1140 1145 1150  
 15 Ile Arg Gly Asn Thr Asn Asp Thr Arg Val Arg Gly Gly Asp Ile Leu  
 1155 1160 1165  
 20 Tyr Phe Asp Met Thr Ile Asn Asn Lys Ala Tyr Asn Leu Phe Met Lys  
 1170 1175 1180  
 Asn Glu Thr Met Tyr Ala Asp Asn His Ser Thr Glu Asp Ile Tyr Ala  
 1185 1190 1195 1200  
 25 Ile Gly Leu Arg Glu Gln Thr Lys Asp Ile Asn Asp Asn Ile Ile Phe  
 1205 1210 1215  
 Gln Ile Gln Pro Met Asn Asn Thr Tyr Tyr Tyr Ala Ser Gln Ile Phe  
 1220 1225 1230  
 30 Lys Ser Asn Phe Asn Gly Glu Asn Ile Ser Gly Ile Cys Ser Ile Gly  
 1235 1240 1245  
 35 Thr Tyr Arg Phe Arg Leu Gly Gly Asp Trp Tyr Arg His Asn Tyr Leu  
 1250 1255 1260  
 Val Pro Thr Val Lys Gln Gly Asn Tyr Ala Ser Leu Leu Glu Ser Thr  
 1265 1270 1275 1280  
 40 Ser Thr His Trp Gly Phe Val Pro Val Ser Glu  
 1285 1290

## (2) INFORMATION FOR SEQ ID NO:61:

45 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 1502 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 50 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:  
 (A) NAME/KEY: CDS  
 55 (B) LOCATION: 108..1493

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:61:

60 AGATCTCGAT CCCGCGAAAT TAATACGACT CACTATAGGG GAATTGTGAG CGGATAACAA 60  
 TTCCCTCTA GAAATAATTT TGTTTAACTT TAAGAAGGAG ATATACC ATG GGC CAT 116  
 Met Gly His  
 1  
 65 CAT CAT CAT CAT CAT CAT CAT CAC AGC AGC GGC CAT ATC GAA GGT 164  
 His His His His His His His His His Ser Ser Gly His Ile Glu Gly  
 5 10 15  
 70 CGT CAT ATG GCT AGC ATG GCT TTA TTA AAA GAT ATA ATT AAT GAA TAT 212  
 Arg His Met Ala Ser Met Ala Leu Leu Lys Asp Ile Ile Asn Glu Tyr

	20	25	30	35	
5	TTC AAT AAT ATT AAT GAT TCA AAA ATT TTG AGC CTA CAA AAC AGA AAA Phe Asn Asn Ile Asn Asp Ser Lys Ile Leu Ser Leu Gln Asn Arg Lys	40	45	50	260
10	AAT ACT TTA GTG GAT ACA TCA GGA TAT AAT GCA GAA GTG AGT GAA GAA Asn Thr Leu Val Asp Thr Ser Gly Tyr Asn Ala Glu Val Ser Glu Glu	55	60	65	308
15	GGC GAT GTT CAG CTT AAT CCA ATA TTT CCA TTT GAC TTT AAA TTA GGT Gly Asp Val Gln Leu Asn Pro Ile Phe Pro Phe Asp Phe Lys Leu Gly	70	75	80	356
20	AGT TCA GGG GAG GAT AGA GGT AAA GTT ATA GTA ACC CAG AAT GAA AAT Ser Ser Gly Glu Asp Arg Gly Lys Val Ile Val Thr Gln Asn Glu Asn	85	90	95	404
25	ATT GTA TAT AAT TCT ATG TAT GAA AGT TTT AGC ATT AGT TTT TGG ATT Ile Val Tyr Asn Ser Met Tyr Glu Ser Phe Ser Ile Ser Phe Trp Ile	100	105	110	452
30	AGA ATA AAT AAA TGG GTA AGT AAT TTA CCT GGA TAT ACT ATA ATT GAT Arg Ile Asn Lys Trp Val Ser Asn Leu Pro Gly Tyr Thr Ile Ile Asp	120	125	130	500
35	AGT GTT AAA AAT AAC TCA GGT TGG AGT ATA GGT ATT ATT AGT AAT TTT Ser Val Lys Asn Asn Ser Gly Trp Ser Ile Gly Ile Ile Ser Asn Phe	135	140	145	548
40	TTA GTA TTT ACT TTA AAA CAA AAT GAA GAT AGT GAA CAA AGT ATA AAT Leu Val Phe Thr Leu Lys Gln Asn Glu Asp Ser Glu Gln Ser Ile Asn	150	155	160	596
45	TTT AGT TAT GAT ATA TCA AAT AAT GCT CCT GGA TAC AAT AAA TGG TTT Phe Ser Tyr Asp Ile Ser Asn Asn Ala Pro Gly Tyr Asn Lys Trp Phe	165	170	175	644
50	TTT GTA ACT GTT ACT AAC AAT ATG ATG GGA AAT ATG AAG ATT TAT ATA Phe Val Thr Val Thr Asn Asn Met Met Gly Asn Met Lys Ile Tyr Ile	180	185	190	692
55	AAT GGA AAA TTA ATA GAT ACT ATA AAA GTT AAA GAA CTA ACT GGA ATT Asn Gly Lys Leu Ile Asp Thr Ile Lys Val Lys Glu Leu Thr Gly Ile	200	205	210	740
60	AAT TTT AGC AAA ACT ATA ACA TTT GAA ATA AAT AAA ATT CCA GAT ACC Asn Phe Ser Lys Thr Ile Thr Phe Glu Ile Asn Lys Ile Pro Asp Thr	215	220	225	788
65	GGT TTG ATT ACT TCA GAT TCT GAT AAC ATC AAT ATG TGG ATA AGA GAT Gly Leu Ile Thr Ser Asp Ser Asp Asn Ile Asn Met Trp Ile Arg Asp	230	235	240	836
70	TTT TAT ATA TTT GCT AAA GAA TTA GAT GGT AAA GAT ATT AAT ATA TTA Phe Tyr Ile Phe Ala Lys Glu Leu Asp Gly Lys Asp Ile Asn Ile Leu	245	250	255	884
75	TTT AAT AGC TTG CAA TAT ACT AAT GTT GTA AAA GAT TAT TGG GGA AAT Phe Asn Ser Leu Gln Tyr Thr Asn Val Val Lys Asp Tyr Trp Gly Asn	260	265	270	932
80	GAT TTA AGA TAT AAT AAA GAA TAT TAT ATG GTT AAT ATA GAT TAT TTA Asp Leu Arg Tyr Asn Lys Glu Tyr Tyr Met Val Asn Ile Asp Tyr Leu	280	285	290	980
85	AAT AGA TAT ATG TAT GCG AAC TCA CGA CAA ATT GTT TTT AAT ACA CGT Asn Arg Tyr Met Tyr Ala Asn Ser Arg Gln Ile Val Phe Asn Thr Arg	295	300	305	1028

	AGA AAT AAT AAT GAC TTC AAT GAA GGA TAT AAA ATT ATA ATA AAA AGA	1076
	Arg Asn Asn Asn Asp Phe Asn Glu Gly Tyr Lys Ile Ile Ile Lys Arg	
	310 315 320	
5	ATC AGA GGA AAT ACA AAT GAT ACT AGA GTA CGA GGA GGA GAT ATT TTA	1124
	Ile Arg Gly Asn Thr Asn Asp Thr Arg Val Arg Gly Gly Asp Ile Leu	
	325 330 335	
10	TAT TTT GAT ATG ACA ATT AAT AAC AAA GCA TAT AAT TTG TTT ATG AAG	1172
	Tyr Phe Asp Met Thr Ile Asn Asn Lys Ala Tyr Asn Leu Phe Met Lys	
	340 345 350 355	
15	AAT GAA ACT ATG TAT GCA GAT AAT CAT AGT ACT GAA GAT ATA TAT GCT	1220
	Asn Glu Thr Met Tyr Ala Asp Asn His Ser Thr Glu Asp Ile Tyr Ala	
	360 365 370	
20	ATA GGT TTA AGA GAA CAA ACA AAG GAT ATA AAT GAT AAT ATT ATA TTT	1268
	Ile Gly Leu Arg Glu Gln Thr Lys Asp Ile Asn Asp Asn Ile Ile Phe	
	375 380 385	
25	CAA ATA CAA CCA ATG AAT AAT ACT TAT TAT TAC GCA TCT CAA ATA TTT	1316
	Gln Ile Gln Pro Met Asn Asn Thr Tyr Tyr Tyr Ala Ser Gln Ile Phe	
	390 395 400	
30	AAA TCA AAT TTT AAT GGA GAA AAT ATT TCT GGA ATA TGT TCA ATA GGT	1364
	Lys Ser Asn Phe Asn Gly Glu Asn Ile Ser Gly Ile Cys Ser Ile Gly	
	405 410 415	
35	ACT TAT CGT TTT AGA CTT GGA GGT GAT TGG TAT AGA CAC AAT TAT TTG	1412
	Thr Tyr Arg Phe Arg Leu Gly Gly Asp Trp Tyr Arg His Asn Tyr Leu	
	420 425 430 435	
40	GTG CCT ACT GTG AAG CAA GGA AAT TAT GCT TCA TTA TTA GAA TCA ACA	1460
	Val Pro Thr Val Lys Gln Gly Asn Tyr Ala Ser Leu Leu Glu Ser Thr	
	440 445 450	
45	TCA ACT CAT TGG GGT TTT GTA CCT GTA AGT GAA TAAAAGCTT	1502
	Ser Thr His Trp Gly Phe Val Pro Val Ser Glu	
	455 460	
	(2) INFORMATION FOR SEQ ID NO:62:	
	(i) SEQUENCE CHARACTERISTICS:	
45	(A) LENGTH: 462 amino acids	
	(B) TYPE: amino acid	
	(D) TOPOLOGY: linear	
	(ii) MOLECULE TYPE: protein	
50	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:62:	
	Met Gly His His His His His His His His His Ser Ser Gly His	
	1 5 10 15	
55	Ile Glu Gly Arg His Met Ala Ser Met Ala Leu Leu Lys Asp Ile Ile	
	20 25 30	
60	Asn Glu Tyr Phe Asn Asn Ile Asn Asp Ser Lys Ile Leu Ser Leu Gln	
	35 40 45	
	Asn Arg Lys Asn Thr Leu Val Asp Thr Ser Gly Tyr Asn Ala Glu Val	
	50 55 60	
65	Ser Glu Glu Gly Asp Val Gln Leu Asn Pro Ile Phe Pro Phe Asp Phe	
	65 70 75 80	
	Lys Leu Gly Ser Ser Gly Glu Asp Arg Gly Lys Val Ile Val Thr Gln	
	85 90 95	
70	Asn Glu Asn Ile Val Tyr Asn Ser Met Tyr Glu Ser Phe Ser Ile Ser	

	100	105	110
	Phe Trp Ile Arg Ile Asn Lys	Trp Val Ser Asn Leu	Pro Gly Tyr Thr
5	115	120	125
	Ile Ile Asp Ser Val Lys Asn Asn Ser Gly Trp Ser Ile Gly Ile Ile		
	130	135	140
10	Ser Asn Phe Leu Val Phe Thr Leu Lys Gln Asn Glu Asp Ser Glu Gln		
	145	150	155
	Ser Ile Asn Phe Ser Tyr Asp Ile Ser Asn Asn Ala Pro Gly Tyr Asn		
	165	170	175
15	Lys Trp Phe Phe Val Thr Val Thr Asn Asn Met Met Gly Asn Met Lys		
	180	185	190
	Ile Tyr Ile Asn Gly Lys Leu Ile Asp Thr Ile Lys Val Lys Glu Leu		
	195	200	205
20	Thr Gly Ile Asn Phe Ser Lys Thr Ile Thr Phe Glu Ile Asn Lys Ile		
	210	215	220
	Pro Asp Thr Gly Leu Ile Thr Ser Asp Ser Asp Asn Ile Asn Met Trp		
	225	230	235
25	Ile Arg Asp Phe Tyr Ile Phe Ala Lys Glu Leu Asp Gly Lys Asp Ile		
	245	250	255
30	Asn Ile Leu Phe Asn Ser Leu Gln Tyr Thr Asn Val Val Lys Asp Tyr		
	260	265	270
	Trp Gly Asn Asp Leu Arg Tyr Asn Lys Glu Tyr Tyr Met Val Asn Ile		
	275	280	285
35	Asp Tyr Leu Asn Arg Tyr Met Tyr Ala Asn Ser Arg Gln Ile Val Phe		
	290	295	300
	Asn Thr Arg Arg Asn Asn Asn Asp Phe Asn Glu Gly Tyr Lys Ile Ile		
	305	310	315
	Ile Lys Arg Ile Arg Gly Asn Thr Asn Asp Thr Arg Val Arg Gly Gly		
	325	330	335
45	Asp Ile Leu Tyr Phe Asp Met Thr Ile Asn Asn Lys Ala Tyr Asn Leu		
	340	345	350
	Phe Met Lys Asn Glu Thr Met Tyr Ala Asp Asn His Ser Thr Glu Asp		
	355	360	365
50	Ile Tyr Ala Ile Gly Leu Arg Glu Gln Thr Lys Asp Ile Asn Asp Asn		
	370	375	380
55	Ile Ile Phe Gln Ile Gln Pro Met Asn Asn Thr Tyr Tyr Tyr Ala Ser		
	385	390	395
	Gln Ile Phe Lys Ser Asn Phe Asn Gly Glu Asn Ile Ser Gly Ile Cys		
	405	410	415
60	Ser Ile Gly Thr Tyr Arg Phe Arg Leu Gly Gly Asp Trp Tyr Arg His		
	420	425	430
	Asn Tyr Leu Val Pro Thr Val Lys Gln Gly Asn Tyr Ala Ser Leu Leu		
	435	440	445
65	Glu Ser Thr Ser Thr His Trp Gly Phe Val Pro Val Ser Glu		
	450	455	460

70) (2) INFORMATION FOR SEQ ID NO:63:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 32 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid  
 (A) DESCRIPTION: /desc = "DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:63:  
 CGCCATGGCT TTATTAAAAG ATATAATTAA TG 32

(2) INFORMATION FOR SEQ ID NO:64:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 32 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid  
 (A) DESCRIPTION: /desc = "DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:64:  
 GCAAGCTTTT ATTCACTTAC AGGTACAAAA CC 32

(2) INFORMATION FOR SEQ ID NO:65:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 3831 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:  
 (A) NAME/KEY: CDS  
 (B) LOCATION: 1..3828

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:65:

45	ATG ACA TGG CCA GTA AAA GAT TTT AAT TAT AGT GAT CCT GTT AAT GAC Met Thr Trp Pro Val Lys Asp Phe Asn Tyr Ser Asp Pro Val Asn Asp 1 5 10 15	48
50	AAT GAT ATA TTA TAT TTA AGA ATA CCA CAA AAT AAG TTA ATT ACT ACA Asn Asp Ile Leu Tyr Leu Arg Ile Pro Gln Asn Lys Leu Ile Thr Thr 20 25 30	96
55	CCT GTA AAA GCT TTT ATG ATT ACT CAA AAT ATT TGG GTA ATA CCA GAA Pro Val Lys Ala Phe Met Ile Thr Gln Asn Ile Trp Val Ile Pro Glu 35 40 45	144
60	AGA TTT TCA TCA GAT ACT AAT CCA AGT TTA AGT AAA CCG CCC AGA CCT Arg Phe Ser Ser Asp Thr Asn Pro Ser Leu Ser Lys Pro Pro Arg Pro 50 55 60	192
65	ACT TCA AAG TAT CAA AGT TAT TAT GAT CCT AGT TAT TTA TCT ACT GAT Thr Ser Lys Tyr Gln Ser Tyr Tyr Asp Pro Ser Tyr Leu Ser Thr Asp 65 70 75 80	240
70	GAA CAA AAA GAT ACA TTT TTA AAA GGG ATT ATA AAA TTA TTT AAA AGA Glu Gln Lys Asp Thr Phe Leu Lys Gly Ile Ile Lys Leu Phe Lys Arg 85 90 95	288
70	ATT AAT GAA AGA GAT ATA GGA AAA AAA TTA ATA AAT TAT TTA GTA GTT Ile Asn Glu Arg Asp Ile Gly Lys Lys Leu Ile Asn Tyr Leu Val Val	336

	100	105	110	
5	GGT TCA CCT TTT ATG GGA GAT TCA AGT ACG CCT GAA GAT ACA TTT GAT Gly Ser Pro Phe Met Gly Asp Ser Ser Thr Pro Glu Asp Thr Phe Asp 115 120 125			384
10	TTT ACA CGT CAT ACT ACT AAT ATT GCA GTT GAA AAG TTT GAA AAT GGT Phe Thr Arg His Thr Thr Asn Ile Ala Val Glu Lys Phe Glu Asn Gly 130 135 140			432
15	AGT TGG AAA GTA ACA AAT ATT ATA ACA CCA AGT GTA TTG ATA TTT GGA Ser Trp Lys Val Thr Ile Ile Thr Pro Ser Val Leu Ile Phe Gly 145 150 155 160			480
20	CCA CTT CCT AAT ATA TTA GAC TAT ACA GCA TCC CTT ACA TTG CAA GGA Pro Leu Pro Asn Ile Leu Asp Tyr Thr Ala Ser Leu Thr Leu Gln Gly 165 170 175			528
25	CAA CAA TCA AAT CCA TCA TTT GAA GGG TTT GGA ACA TTA TCT ATA CTA Gln Gln Ser Asn Pro Ser Phe Glu Gly Phe Gly Thr Leu Ser Ile Leu 180 185 190			576
30	AAA GTA GCA CCT GAA TTT TTG TTA ACA TTT AGT GAT GTA ACA TCT AAT Lys Val Ala Pro Glu Phe Leu Leu Thr Phe Ser Asp Val Thr Ser Asn 195 200 205			624
35	CAA AGT TCA GCT GTA TTA GGC AAA TCT ATA TTT TGT ATG GAT CCA GTA Gln Ser Ser Ala Val Leu Gly Lys Ser Ile Phe Cys Met Asp Pro Val 210 215 220			672
40	ATA GCT TTA ATG CAT GAG TTA ACA CAT TCT TTG CAT CAA TTA TAT GGA Ile Ala Leu Met His Glu Leu Thr His Ser Leu His Gln Leu Tyr Gly 225 230 235 240			720
45	ATA AAT ATA CCA TCT GAT AAA AGG ATT CGT CCA CAA GTT AGC GAG GGA Ile Asn Ile Pro Ser Asp Lys Arg Ile Arg Pro Gln Val Ser Glu Gly 245 250 255			768
50	TTT TTC TCT CAA GAT GGA CCC AAC GTA CAA TTT GAG GAA TTA TAT ACA Phe Phe Ser Gln Asp Gly Pro Asn Val Gln Phe Glu Glu Leu Tyr Thr 260 265 270			816
55	TTT GGA GGA TTA GAT GTT GAA ATA ATA CCT CAA ATT GAA AGA TCA CAA Phe Gly Gly Leu Asp Val Glu Ile Ile Pro Gln Ile Glu Arg Ser Gln 275 280 285			864
60	TTA AGA GAA AAA GCA TTA GGT CAC TAT AAA GAT ATA GCG AAA AGA CTT Leu Arg Glu Lys Ala Leu Gly His Tyr Lys Asp Ile Ala Lys Arg Leu 290 295 300			912
65	AAT AAT ATT AAT AAA ACT ATT CCT TCT AGT TGG ATT AGT AAT ATA GAT Asn Asn Ile Asn Lys Thr Ile Pro Ser Ser Trp Ile Ser Asn Ile Asp 305 310 315 320			960
70	AAA TAT AAA AAA ATA TTT TCT GAA AAG TAT AAT TTT GAT AAA GAT AAT Lys Tyr Lys Lys Ile Phe Ser Glu Lys Tyr Asn Phe Asp Lys Asp Asn 325 330 335			1008
75	ACA GGA AAT TTT GTT GTA AAT ATT GAT AAA TTC AAT AGC TTA TAT TCA Thr Gly Asn Phe Val Val Asn Ile Asp Lys Phe Asn Ser Leu Tyr Ser 340 345 350			1056

	GAC TTG ACT AAT GTT ATG TCA GAA GTT GTT TAT TCT TCG CAA TAT AAT Asp Leu Thr Asn Val Met Ser Glu Val Val Tyr Ser Ser Gln Tyr Asn	1104
	355 360 365	
5	GTT AAA AAC AGG ACT CAT TAT TTT TCA AGG CAT TAT CTA CCT GTA TTT Val Lys Asn Arg Thr His Tyr Phe Ser Arg His Tyr Leu Pro Val Phe	1152
	370 375 380	
10	GCA AAT ATA TTA GAT GAT AAT ATT TAT ACT ATA AGA GAT GGT TTT AAT Ala Asn Ile Leu Asp Asp Asn Ile Tyr Thr Ile Arg Asp Gly Phe Asn	1200
	385 390 395 400	
15	TTA ACA AAT AAA GGT TTT AAT ATA GAA AAT TCG GGT CAG AAT ATA GAA Leu Thr Asn Lys Gly Phe Asn Ile Glu Asn Ser Gly Gln Asn Ile Glu	1248
	405 410 415	
20	AGG AAT CCT GCA CTA CAA AAG CTT AGT TCA GAA AGT GTA GTA GAT TTA Arg Asn Pro Ala Leu Gln Lys Leu Ser Ser Glu Ser Val Val Asp Leu	1296
	420 425 430	
	TTT ACA AAA GTA TGT TTA AGA TTA ACA AAA AAT AGT AGA GAT GAT TCA Phe Thr Lys Val Cys Leu Arg Leu Thr Lys Asn Ser Arg Asp Asp Ser	1344
	435 440 445	
25	ACA TGT ATT AAA GTT AAA AAT AAT AGA TTA CCT TAT GTA GCT GAT AAA Thr Cys Ile Lys Val Lys Asn Asn Arg Leu Pro Tyr Val Ala Asp Lys	1392
	450 455 460	
30	GAT AGC ATT TCA CAA GAA ATA TTT GAA AAT AAA ATT ATT ACA GAT GAG Asp Ser Ile Ser Gln Glu Ile Phe Glu Asn Lys Ile Ile Thr Asp Glu	1440
	465 470 475 480	
35	ACT AAT GTA CAA AAT TAT TCA GAT AAT TTT TCA TTA GAT GAA TCT ATT Thr Asn Val Gln Asn Tyr Ser Asp Asn Phe Ser Leu Asp Glu Ser Ile	1488
	485 490 495	
40	TTA GAT GGG CAA GTT CCT ATT AAT CCT GAA ATA GTA GAT CCA CTA TTA Leu Asp Gly Gln Val Pro Ile Asn Pro Glu Ile Val Asp Pro Leu Leu	1536
	500 505 510	
	CCC AAT GTT AAT ATG GAA CCT TTA AAT CTT CCA GGT GAA GAA ATA GTA Pro Asn Val Asn Met Glu Pro Leu Asn Leu Pro Gly Glu Glu Ile Val	1584
	515 520 525	
45	TTT TAT GAT GAT ATT ACT AAA TAT GTT GAT TAT TTA AAT TCT TAT TAT Phe Tyr Asp Asp Ile Thr Lys Tyr Val Asp Tyr Leu Asn Ser Tyr Tyr	1632
	530 535 540	
50	TAT TTG GAA TCT CAA AAA TTA AGT AAT AAT GTT GAA AAT ATT ACT CTT Tyr Leu Glu Ser Gln Lys Leu Ser Asn Asn Val Glu Asn Ile Thr Leu	1680
	545 550 555 560	
55	ACA ACT TCA GTT GAA GAA GCA TTA GGT TAT AGC AAT AAG ATA TAC ACA Thr Thr Ser Val Glu Glu Ala Leu Gly Tyr Ser Asn Lys Ile Tyr Thr	1728
	565 570 575	
60	TTT TTA CCT AGC TTA GCT GAA AAA GTG AAT AAA GGT GTT CAA GCA GGT Phe Leu Pro Ser Leu Ala Glu Lys Val Asn Lys Gly Val Gln Ala Gly	1776
	580 585 590	
	TTA TTC TTA AAT TGG GCG AAT GAA GTA GTT GAG GAT TTT ACT ACA AAT Leu Phe Leu Asn Trp Ala Asn Glu Val Val Glu Asp Phe Thr Thr Asn	1824
	595 600 605	
65	ATT ATG AAG AAA GAT ACA TTG GAT AAA ATA TCA GAT GTA TCA GTA ATA Ile Met Lys Lys Asp Thr Leu Asp Lys Ile Ser Asp Val Ser Val Ile	1872
	610 615 620	
70	ATT CCA TAT ATA GGA CCT GCC TTA AAT ATA GGA AAT TCA GCA TTA AGG Ile Pro Tyr Ile Gly Pro Ala Leu Asn Ile Gly Asn Ser Ala Leu Arg	1920

	625	630	635	640	
5	GGA AAT TTT AAG CAA GCA TTT GCA ACA GCT GGT GTA GCT TTT TTA TTA Gly Asn Phe Lys 645 Ala Phe Ala Thr Ala Gly Val Ala Phe Leu Leu 655	1968			
10	GAG GGA TTT CCA GAG TTT ACT ATA CCT GCA CTC GGT GTA TTT ACC TTT Glu Gly Phe Pro 660 Glu Phe Thr Ile Pro Ala Leu Gly Val Phe Thr Phe 665 670	2016			
15	TAT AGT TCT ATT CAA GAA AGA GAG AAA ATT ATT AAA ACT ATA GAA AAT Tyr Ser Ser Ile 675 Gln Glu Arg Glu Lys Ile Ile Lys Thr Ile Glu Asn 680 685	2064			
20	TGT TTG GAA CAA AGA GTT AAG AGA TGG AAA GAT TCA TAT CAA TGG ATG Cys Leu Glu Gln Arg Val 695 Lys Arg Trp Lys Asp Ser Tyr Gln Trp Met 700	2112			
25	GTA TCA AAT TGG TTG TCA AGA ATT ACT ACT CAA TTT AAT CAT ATA AAT Val Ser Asn Trp Leu Ser Arg Ile Thr Thr Gln Phe Asn His Ile Asn 705 710 715 720	2160			
30	TAT CAA ATG TAT GAT TCT TTA AGT TAT CAG GCA GAT GCA ATC AAA GCT Tyr Gln Met Tyr 725 Asp Ser Leu Ser Tyr Gln Ala Asp Ala Ile Lys Ala 730 735	2208			
35	AAA ATA GAT TTA GAA TAT AAA AAA TAC TCA GGA AGT GAT AAA GAA AAT Lys Ile Asp Leu Glu Tyr Lys Lys Tyr Ser Gly Ser Asp Lys Glu Asn 740 745 750	2256			
40	ATA AAA AGT CAA GTT GAA AAT TTA AAA AAT AGT TTA GAT GTA AAA ATT Ile Lys Ser Gln Val Glu Asn Leu Lys Asn Ser Leu Asp Val Lys Ile 755 760 765	2304			
45	TCG GAA GCA ATG AAT AAT ATA AAT AAA TTT ATA CGA GAA TGT TCT GTA Ser Glu Ala Met Asn Asn Ile Asn Lys Phe Ile Arg Glu Cys Ser Val 770 775 780	2352			
50	ACA TAC TTA TTT AAA AAT ATG CTC CCT AAA GTA ATT GAC GAA TTA AAT Thr Tyr Leu Phe Lys Asn Met Leu Pro Lys Val Ile Asp Glu Leu Asn 785 790 795 800	2400			
55	AAG TTT GAT TTA AGA ACT AAA ACA GAA TTA ATT AAT CTT ATA GAT AGT Lys Phe Asp Leu Arg Thr Lys Thr Glu Leu Ile Asn Leu Ile Asp Ser 805 810 815	2448			
60	CAT AAT ATT ATT CTA GTT GGT GAA GTA GAT AGA TTA AAA GCA AAA GTA His Asn Ile Ile Leu Val Gly Glu Val Asp Arg Leu Lys Ala Lys Val 820 825 830	2496			
65	AAT GAG AGT TTT GAA AAT ACA ATG CCT TTT AAT ATT TTT TCA TAT ACT Asn Glu Ser Phe Glu Asn Thr Met Pro Phe Asn Ile Phe Ser Tyr Thr 835 840 845	2544			
70	AAT AAT TCT TTA TTA AAA GAT ATA ATT AAT GAA TAT TTC AAT AGT ATT Asn Asn Ser Leu Leu Lys Asp Ile Ile Asn Glu Tyr Phe Asn Ser Ile 850 855 860	2592			
75	AAT GAT TCA AAA ATT TTG AGC TTA CAA AAC AAA AAA AAT GCT TTA GTG Asn Asp Ser Lys Ile Leu Ser Leu Gln Asn Lys Lys Asn Ala Leu Val 865 870 875 880	2640			
80	GAT ACA TCA GGA TAT AAT GCA GAA GTG AGG GTA GGA GAT AAT GTT CAA Asp Thr Ser Gly Tyr Asn Ala Glu Val Arg Val Gly Asp Asn Val Gln 885 890 895	2688			
85	CTT AAT ACG ATA TAT ACA AAT GAC TTT AAA TTA AGT AGT TCA GGA GAT Leu Asn Thr Ile Tyr Thr Asn Asp Phe Lys Leu Ser Ser Ser Gly Asp 900 905 910	2736			



	AAA ATT ATA GTA AAT TTA AAT AAT AAT ATT TTA TAT AGC GCT ATT TAT Lys Ile Ile Val Asn Leu Asn Asn Asn Ile Leu Tyr Ser Ala Ile Tyr 915 920 925	2784
5	GAG AAC TCT AGT GTT AGT TTT TGG ATT AAG ATA TCT AAA GAT TTA ACT Glu Asn Ser Ser Val Ser Phe Trp Ile Lys Ile Ser Lys Asp Leu Thr 930 935 940	2832
10	AAT TCT CAT AAT GAA TAT ACA ATA ATT AAC AGT ATA GAA CAA AAT TCT Asn Ser His Asn Glu Tyr Thr Ile Ile Asn Ser Ile Glu Gln Asn Ser 945 950 955 960	2880
15	GGG TGG AAA TTA TGT ATT AGG AAT GGC AAT ATA GAA TGG ATT TTA CAA Gly Trp Lys Leu Cys Ile Arg Asn Gly Asn Ile Glu Trp Ile Leu Gln 965 970 975	2928
20	GAT GTT AAT AGA AAG TAT AAA AGT TTA ATT TTT GAT TAT AGT GAA TCA Asp Val Asn Arg Lys Tyr Lys Ser Leu Ile Phe Asp Tyr Ser Glu Ser 980 985 990	2976
	TTA AGT CAT ACA GGA TAT ACA AAT AAA TGG TTT TTT GTT ACT ATA ACT Leu Ser His Thr Gly Tyr Thr Asn Lys Trp Phe Phe Val Thr Ile Thr 995 1000 1005	3024
25	AAT AAT ATA ATG GGG TAT ATG AAA CTT TAT ATA AAT GGA GAA TTA AAG Asn Asn Ile Met Gly Tyr Met Lys Leu Tyr Ile Asn Gly Glu Leu Lys 1010 1015 1020	3072
30	CAG AGT CAA AAA ATT GAA GAT TTA GAT GAG GTT AAG TTA GAT AAA ACC Gln Ser Gln Lys Ile Glu Asp Leu Asp Glu Val Lys Leu Asp Lys Thr 1025 1030 1035 1040	3120
35	ATA GTA TTT GGA ATA GAT GAG AAT ATA GAT GAG AAT CAG ATG CTT TGG Ile Val Phe Gly Ile Asp Glu Asn Ile Asp Glu Asn Gln Met Leu Trp 1045 1050 1055	3168
40	ATT AGA GAT TTT AAT ATT TTT TCT AAA GAA TTA AGT AAT GAA GAT ATT Ile Arg Asp Phe Asn Ile Phe Ser Lys Glu Leu Ser Asn Glu Asp Ile 1060 1065 1070	3216
	AAT ATT GTA TAT GAG GGA CAA ATA TTA AGA AAT GTT ATT AAA GAT TAT Asn Ile Val Tyr Glu Gly Gln Ile Leu Arg Asn Val Ile Lys Asp Tyr 1075 1080 1085	3264
45	TGG GGA AAT CCT TTC AAG TTT GAT ACA GAA TAT TAT ATT ATT AAT GAT Trp Gly Asn Pro Leu Lys Phe Asp Thr Glu Tyr Tyr Ile Ile Asn Asp 1090 1095 1100	3312
50	AAT TAT ATA GAT AGG TAT ATT GCA CCT GAA AGT AAT GTA CTT GTA CTT Asn Tyr Ile Asp Arg Tyr Ile Ala Pro Glu Ser Asn Val Leu Val Leu 1105 1110 1115 1120	3360
55	GTT CGG TAT CCA GAT AGA TCT AAA TTA TAT ACT GGA AAT CCT ATT ACT Val Arg Tyr Pro Asp Arg Ser Lys Leu Tyr Thr Gly Asn Pro Ile Thr 1125 1130 1135	3408
60	ATT AAA TCA GTA TCT GAT AAG AAT CCT TAT AGT AGA ATT TTA AAT GGA Ile Lys Ser Val Ser Asp Lys Asn Pro Tyr Ser Arg Ile Leu Asn Gly 1140 1145 1150	3456
	GAT AAT ATA ATT CTT CAT ATG TTA TAT AAT AGT AGG AAA TAT ATG ATA Asp Asn Ile Ile Leu His Met Leu Tyr Asn Ser Arg Lys Tyr Met Ile 1155 1160 1165	3504
65	ATA AGA GAT ACT GAT ACA ATA TAT GCA ACA CAA GGA GGA GAG TGT TCA Ile Arg Asp Thr Asp Thr Ile Tyr Ala Thr Gln Gly Gly Glu Cys Ser 1170 1175 1180	3552
70	CAA AAT TGT GTA TAT GCA TTA AAA TTA CAG AGT AAT TTA GGT AAT TAT Gln Asn Cys Val Tyr Ala Leu Lys Leu Gln Ser Asn Leu Gly Asn Tyr	3600

	1185	1190	1195	1200	
5	GGT ATA GGT ATA TTT AGT ATA AAA AAT ATT GTA TCT AAA AAT AAA TAT Gly Ile Gly Ile Phe Ser Ile Lys Asn Ile Val Ser Lys Asn Lys Tyr 1205 1210 1215	3648			
10	TGT AGT CAA ATT TTC TCT AGT TTT AGG GAA AAT ACA ATG CTT CTA GCA Cys Ser Gln Ile Phe Ser Ser Phe Arg Glu Asn Thr Met Leu Leu Ala 1220 1225 1230	3696			
15	GAT ATA TAT AAA CCT TGG AGA TTT TCT TTT AAA AAT GCA TAC ACG CCA Asp Ile Tyr Lys Pro Trp Arg Phe Ser Phe Lys Asn Ala Tyr Thr Pro 1235 1240 1245	3744			
20	GTT GCA GTA ACT AAT TAT GAA ACA AAA CTA TTA TCA ACT TCA TCT TTT Val Ala Val Thr Asn Tyr Glu Thr Lys Leu Leu Ser Thr Ser Ser Phe 1250 1255 1260	3792			
25	TGG AAA TTT ATT TCT AGG GAT CCA GGA TGG GTA GAG TAA Trp Lys Phe Ile Ser Arg Asp Pro Gly Trp Val Glu 1265 1270 1275	3831			
	(2) INFORMATION FOR SEQ ID NO:66:				
25	(i) SEQUENCE CHARACTERISTICS:				
	(A) LENGTH: 1276 amino acids				
	(B) TYPE: amino acid				
	(D) TOPOLOGY: linear				
30	(ii) MOLECULE TYPE: protein				
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:66:				
35	Met Thr Trp Pro Val Lys Asp Phe Asn Tyr Ser Asp Pro Val Asn Asp 1 5 10 15				
	Asn Asp Ile Leu Tyr Leu Arg Ile Pro Gln Asn Lys Leu Ile Thr Thr 20 25 30				
40	Pro Val Lys Ala Phe Met Ile Thr Gln Asn Ile Trp Val Ile Pro Glu 35 40 45				
45	Arg Phe Ser Ser Asp Thr Asn Pro Ser Leu Ser Lys Pro Pro Arg Pro 50 55 60				
	Thr Ser Lys Tyr Gln Ser Tyr Tyr Asp Pro Ser Tyr Leu Ser Thr Asp 65 70 75 80				
50	Glu Gln Lys Asp Thr Phe Leu Lys Gly Ile Ile Lys Leu Phe Lys Arg 85 90 95				
	Ile Asn Glu Arg Asp Ile Gly Lys Lys Leu Ile Asn Tyr Leu Val Val 100 105 110				
55	Gly Ser Pro Phe Met Gly Asp Ser Ser Thr Pro Glu Asp Thr Phe Asp 115 120 125				
60	Phe Thr Arg His Thr Thr Asn Ile Ala Val Glu Lys Phe Glu Asn Gly 130 135 140				
	Ser Trp Lys Val Thr Asn Ile Ile Thr Pro Ser Val Leu Ile Phe Gly 145 150 155 160				
65	Pro Leu Pro Asn Ile Leu Asp Tyr Thr Ala Ser Leu Thr Leu Gln Gly 165 170 175				
	Gln Gln Ser Asn Pro Ser Phe Glu Gly Phe Gly Thr Leu Ser Ile Leu 180 185 190				
70	Lys Val Ala Pro Glu Phe Leu Leu Thr Phe Ser Asp Val Thr Ser Asn				

	195	200	205
5	Gln Ser Ser Ala Val Leu Gly Lys Ser Ile Phe Cys Met Asp Pro Val 210 215 220		
	Ile Ala Leu Met His Glu Leu Thr His Ser Leu His Gln Leu Tyr Gly 225 230 235 240		
10	Ile Asn Ile Pro Ser Asp Lys Arg Ile Arg Pro Gln Val Ser Glu Gly 245 250 255		
	Phe Phe Ser Gln Asp Gly Pro Asn Val Gln Phe Glu Glu Leu Tyr Thr 260 265 270		
15	Phe Gly Gly Leu Asp Val Glu Ile Ile Pro Gln Ile Glu Arg Ser Gln 275 280 285		
20	Leu Arg Glu Lys Ala Leu Gly His Tyr Lys Asp Ile Ala Lys Arg Leu 290 295 300		
	Asn Asn Ile Asn Lys Thr Ile Pro Ser Ser Trp Ile Ser Asn Ile Asp 305 310 315 320		
25	Lys Tyr Lys Lys Ile Phe Ser Glu Lys Tyr Asn Phe Asp Lys Asp Asn 325 330 335		
	Thr Gly Asn Phe Val Val Asn Ile Asp Lys Phe Asn Ser Leu Tyr Ser 340 345 350		
30	Asp Leu Thr Asn Val Met Ser Glu Val Val Tyr Ser Ser Gln Tyr Asn 355 360 365		
35	Val Lys Asn Arg Thr His Tyr Phe Ser Arg His Tyr Leu Pro Val Phe 370 375 380		
	Ala Asn Ile Leu Asp Asp Asn Ile Tyr Thr Ile Arg Asp Gly Phe Asn 385 390 395 400		
40	Leu Thr Asn Lys Gly Phe Asn Ile Glu Asn Ser Gly Gln Asn Ile Glu 405 410 415		
	Arg Asn Pro Ala Leu Gln Lys Leu Ser Ser Glu Ser Val Val Asp Leu 420 425 430		
45	Phe Thr Lys Val Cys Leu Arg Leu Thr Lys Asn Ser Arg Asp Asp Ser 435 440 445		
50	Thr Cys Ile Lys Val Lys Asn Asn Arg Leu Pro Tyr Val Ala Asp Lys 450 455 460		
	Asp Ser Ile Ser Gln Glu Ile Phe Glu Asn Lys Ile Ile Thr Asp Glu 465 470 475 480		
55	Thr Asn Val Gln Asn Tyr Ser Asp Asn Phe Ser Leu Asp Glu Ser Ile 485 490 495		
	Leu Asp Gly Gln Val Pro Ile Asn Pro Glu Ile Val Asp Pro Leu Leu 500 505 510		
60	Pro Asn Val Asn Met Glu Pro Leu Asn Leu Pro Gly Glu Glu Ile Val 515 520 525		
65	Phe Tyr Asp Asp Ile Thr Lys Tyr Val Asp Tyr Leu Asn Ser Tyr Tyr 530 535 540		
	Tyr Leu Glu Ser Gln Lys Leu Ser Asn Asn Val Glu Asn Ile Thr Leu 545 550 555 560		
70	Thr Thr Ser Val Glu Glu Ala Leu Gly Tyr Ser Asn Lys Ile Tyr Thr 565 570 575		

Phe Leu Pro Ser Leu Ala Glu Lys Val Asn Lys Gly Val Gln Ala Gly  
 580 585 590  
 5 Leu Phe Leu Asn Trp Ala Asn Glu Val Val Glu Asp Phe Thr Thr Asn  
 595 600 605  
 Ile Met Lys Lys Asp Thr Leu Asp Lys Ile Ser Asp Val Ser Val Ile  
 610 615 620  
 10 Ile Pro Tyr Ile Gly Pro Ala Leu Asn Ile Gly Asn Ser Ala Leu Arg  
 625 630 635 640  
 Gly Asn Phe Lys Gln Ala Phe Ala Thr Ala Gly Val Ala Phe Leu Leu  
 645 650 655  
 15 Glu Gly Phe Pro Glu Phe Thr Ile Pro Ala Leu Gly Val Phe Thr Phe  
 660 665 670  
 20 Tyr Ser Ser Ile Gln Glu Arg Glu Lys Ile Ile Lys Thr Ile Glu Asn  
 675 680 685  
 Cys Leu Glu Gln Arg Val Lys Arg Trp Lys Asp Ser Tyr Gln Trp Met  
 690 695 700  
 25 Val Ser Asn Trp Leu Ser Arg Ile Thr Thr Gln Phe Asn His Ile Asn  
 705 710 715 720  
 Tyr Gln Met Tyr Asp Ser Leu Ser Tyr Gln Ala Asp Ala Ile Lys Ala  
 725 730 735  
 30 Lys Ile Asp Leu Glu Tyr Lys Lys Tyr Ser Gly Ser Asp Lys Glu Asn  
 740 745 750  
 35 Ile Lys Ser Gln Val Glu Asn Leu Lys Asn Ser Leu Asp Val Lys Ile  
 755 760 765  
 Ser Glu Ala Met Asn Asn Ile Asn Lys Phe Ile Arg Glu Cys Ser Val  
 770 775 780  
 40 Thr Tyr Leu Phe Lys Asn Met Leu Pro Lys Val Ile Asp Glu Leu Asn  
 785 790 795 800  
 Lys Phe Asp Leu Arg Thr Lys Thr Glu Leu Ile Asn Leu Ile Asp Ser  
 805 810 815  
 45 His Asn Ile Ile Leu Val Gly Glu Val Asp Arg Leu Lys Ala Lys Val  
 820 825 830  
 50 Asn Glu Ser Phe Glu Asn Thr Met Pro Phe Asn Ile Phe Ser Tyr Thr  
 835 840 845  
 Asn Asn Ser Leu Leu Lys Asp Ile Ile Asn Glu Tyr Phe Asn Ser Ile  
 850 855 860  
 55 Asn Asp Ser Lys Ile Leu Ser Leu Gln Asn Lys Lys Asn Ala Leu Val  
 865 870 875 880  
 Asp Thr Ser Gly Tyr Asn Ala Glu Val Arg Val Gly Asp Asn Val Gln  
 885 890 895  
 60 Leu Asn Thr Ile Tyr Thr Asn Asp Phe Lys Leu Ser Ser Ser Gly Asp  
 900 905 910  
 65 Lys Ile Ile Val Asn Leu Asn Asn Ile Leu Tyr Ser Ala Ile Tyr  
 915 920 925  
 Glu Asn Ser Ser Val Ser Phe Trp Ile Lys Ile Ser Lys Asp Leu Thr  
 930 935 940  
 70 Asn Ser His Asn Glu Tyr Thr Ile Ile Asn Ser Ile Glu Gln Asn Ser

	945		950		955		960
	Gly Trp Lys Leu Cys	Ile Arg Asn Gly Asn	Ile Glu Trp Ile Leu Gln				
5		965	970			975	
	Asp Val Asn Arg Lys Tyr Lys Ser Leu Ile Phe Asp Tyr Ser Glu Ser		985			990	
		980					
10	Leu Ser His Thr Gly Tyr Thr Asn Lys Trp Phe Phe Val Thr Ile Thr		1000			1005	
		995					
	Asn Asn Ile Met Gly Tyr Met Lys Leu Tyr Ile Asn Gly Glu Leu Lys		1015			1020	
		1010					
15	Gln Ser Gln Lys Ile Glu Asp Leu Asp Glu Val Lys Leu Asp Lys Thr		1030			1035	1040
	Ile Val Phe Gly Ile Asp Glu Asn Ile Asp Glu Asn Gln Met Leu Trp		1045			1050	1055
20							
	Ile Arg Asp Phe Asn Ile Phe Ser Lys Glu Leu Ser Asn Glu Asp Ile		1060			1065	1070
25	Asn Ile Val Tyr Glu Gly Gln Ile Leu Arg Asn Val Ile Lys Asp Tyr		1075			1080	1085
	Trp Gly Asn Pro Leu Lys Phe Asp Thr Glu Tyr Tyr Ile Ile Asn Asp		1090			1095	1100
30	Asn Tyr Ile Asp Arg Tyr Ile Ala Pro Glu Ser Asn Val Leu Val Leu		1105			1110	1115
	Val Arg Tyr Pro Asp Arg Ser Lys Leu Tyr Thr Gly Asn Pro Ile Thr		1125			1130	1135
35							
	Ile Lys Ser Val Ser Asp Lys Asn Pro Tyr Ser Arg Ile Leu Asn Gly		1140			1145	1150
40	Asp Asn Ile Ile Leu His Met Leu Tyr Asn Ser Arg Lys Tyr Met Ile		1155			1160	1165
	Ile Arg Asp Thr Asp Thr Ile Tyr Ala Thr Gln Gly Gly Glu Cys Ser		1170			1175	1180
45	Gln Asn Cys Val Tyr Ala Leu Lys Leu Gln Ser Asn Leu Gly Asn Tyr		1185			1190	1195
	Gly Ile Gly Ile Phe Ser Ile Lys Asn Ile Val Ser Lys Asn Lys Tyr		1205			1210	1215
50							
	Cys Ser Gln Ile Phe Ser Ser Phe Arg Glu Asn Thr Met Leu Leu Ala		1220			1225	1230
55	Asp Ile Tyr Lys Pro Trp Arg Phe Ser Phe Lys Asn Ala Tyr Thr Pro		1235			1240	1245
	Val Ala Val Thr Asn Tyr Glu Thr Lys Leu Leu Ser Thr Ser Ser Phe		1250			1255	1260
60	Trp Lys Phe Ile Ser Arg Asp Pro Gly Trp Val Glu		1265			1270	1275

## (2) INFORMATION FOR SEQ ID NO:67:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1469 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (ix) FEATURE:

- (A) NAME/KEY: CDS  
 (B) LOCATION: 108..1460

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:67:

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AGATCTCGAT CCCGCGAAAT TAATACGACT CACTATAGGG GAATTGTGAG CGGATAACAA      60
TTCCCCCTCTA GAAATAATTT TGTTTAACTT TAAGAAGGAG ATATACC ATG GGC CAT      116
                                     Met Gly His
                                     1
CAT CAT CAT CAT CAT CAT CAT CAT CAC AGC AGC GGC CAT ATC GAA GGT      164
His His His His His His His His His Ser Ser Gly His Ile Glu Gly
      5                                10                                15
CGT CAT ATG GCT AGC ATG GCT TTA TTA AAA GAT ATA ATT AAT GAA TAT      212
Arg His Met Ala Ser Met Ala Leu Leu Lys Asp Ile Ile Asn Glu Tyr
      20                                25                                30
TTC AAT AGT ATT AAT GAT TCA AAA ATT TTG AGC TTA CAA AAC AAA AAA      260
Phe Asn Ser Ile Asn Asp Ser Lys Ile Leu Ser Leu Gln Asn Lys Lys
      40                                45                                50
AAT GCT TTA GTG GAT ACA TCA GGA TAT AAT GCA GAA GTG AGG GTA GGA      308
Asn Ala Leu Val Asp Thr Ser Gly Tyr Asn Ala Glu Val Arg Val Gly
      55                                60                                65
GAT AAT GTT CAA CTT AAT ACG ATA TAT ACA AAT GAC TTT AAA TTA AGT      356
Asp Asn Val Gln Leu Asn Thr Ile Tyr Thr Asn Asp Phe Lys Leu Ser
      70                                75                                80
AGT TCA GGA GAT AAA ATT ATA GTA AAT TTA AAT AAT AAT ATT TTA TAT      404
Ser Ser Gly Asp Lys Ile Ile Val Asn Leu Asn Asn Asn Ile Leu Tyr
      85                                90                                95
AGC GCT ATT TAT GAG AAC TCT AGT GTT AGT TTT TGG ATT AAG ATA TCT      452
Ser Ala Ile Tyr Glu Asn Ser Ser Val Ser Phe Trp Ile Lys Ile Ser
      100                               105                               110                               115
AAA GAT TTA ACT AAT TCT CAT AAT GAA TAT ACA ATA ATT AAC AGT ATA      500
Lys Asp Leu Thr Asn Ser His Asn Glu Tyr Thr Ile Ile Asn Ser Ile
      120                               125                               130
GAA CAA AAT TCT GGG TGG AAA TTA TGT ATT AGG AAT GGC AAT ATA GAA      548
Glu Gln Asn Ser Gly Trp Lys Leu Cys Ile Arg Asn Gly Asn Ile Glu
      135                               140                               145
TGG ATT TTA CAA GAT GTT AAT AGA AAG TAT AAA AGT TTA ATT TTT GAT      596
Trp Ile Leu Gln Asp Val Asn Arg Lys Tyr Lys Ser Leu Ile Phe Asp
      150                               155                               160
TAT AGT GAA TCA TTA AGT CAT ACA GGA TAT ACA AAT AAA TGG TTT TTT      644
Tyr Ser Glu Ser Leu Ser His Thr Gly Tyr Thr Asn Lys Trp Phe Phe
      165                               170                               175
GTT ACT ATA ACT AAT AAT ATA ATG GGG TAT ATG AAA CTT TAT ATA AAT      692
Val Thr Ile Thr Asn Asn Ile Met Gly Tyr Met Lys Leu Tyr Ile Asn
      180                               185                               190                               195

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	GGA GAA TTA AAG CAG AGT CAA AAA ATT GAA GAT TTA GAT GAG GTT AAG Gly Glu Leu Lys Gln Ser Gln Lys Ile Glu Asp Leu Asp Glu Val Lys	740
5	TTA GAT AAA ACC ATA GTA TTT GGA ATA GAT GAG AAT ATA GAT GAG AAT Leu Asp Lys Thr Ile Val Phe Gly Ile Asp Glu Asn Ile Asp Glu Asn	788
10	CAG ATG CTT TGG ATT AGA GAT TTT AAT ATT TTT TCT AAA GAA TTA AGT Gln Met Leu Trp Ile Arg Asp Phe Asn Ile Phe Ser Lys Glu Leu Ser	836
15	AAT GAA GAT ATT AAT ATT GTA TAT GAG GGA CAA ATA TTA AGA AAT GTT Asn Glu Asp Ile Asn Ile Val Tyr Glu Gly Gln Ile Leu Arg Asn Val	884
20	ATT AAA GAT TAT TGG GGA AAT CCT TTG AAG TTT GAT ACA GAA TAT TAT Ile Lys Asp Tyr Trp Gly Asn Pro Leu Lys Phe Asp Thr Glu Tyr Tyr	932
25	ATT ATT AAT GAT AAT TAT ATA GAT AGG TAT ATT GCA CCT GAA AGT AAT Ile Ile Asn Asp Asn Tyr Ile Asp Arg Tyr Ile Ala Pro Glu Ser Asn	980
30	GTA CTT GTA CTT GTT CGG TAT CCA GAT AGA TCT AAA TTA TAT ACT GGA Val Leu Val Leu Val Arg Tyr Pro Asp Arg Ser Lys Leu Tyr Thr Gly	1028
35	AAT CCT ATT ACT ATT AAA TCA GTA TCT GAT AAG AAT CCT TAT AGT AGA Asn Pro Ile Thr Ile Lys Ser Val Ser Asp Lys Asn Pro Tyr Ser Arg	1076
40	ATT TTA AAT GGA GAT AAT ATA ATT CTT CAT ATG TTA TAT AAT AGT AGG Ile Leu Asn Gly Asp Asn Ile Ile Leu His Met Leu Tyr Asn Ser Arg	1124
45	AAA TAT ATG ATA ATA AGA GAT ACT GAT ACA ATA TAT GCA ACA CAA GGA Lys Tyr Met Ile Ile Arg Asp Thr Asp Thr Ile Tyr Ala Thr Gln Gly	1172
50	GGA GAG TGT TCA CAA AAT TGT GTA TAT GCA TTA AAA TTA CAG AGT AAT Gly Glu Cys Ser Gln Asn Cys Val Tyr Ala Leu Lys Leu Gln Ser Asn	1220
55	TTA GGT AAT TAT GGT ATA GGT ATA TTT AGT ATA AAA AAT ATT GTA TCT Leu Gly Asn Tyr Gly Ile Gly Ile Phe Ser Ile Lys Asn Ile Val Ser	1268
60	AAA AAT AAA TAT TGT AGT CAA ATT TTC TCT AGT TTT AGG GAA AAT ACA Lys Asn Lys Tyr Cys Ser Gln Ile Phe Ser Ser Phe Arg Glu Asn Thr	1316
65	ATG CTT CTA GCA GAT ATA TAT AAA CCT TGG AGA TTT TCT TTT AAA AAT Met Leu Leu Ala Asp Ile Tyr Lys Pro Trp Arg Phe Ser Phe Lys Asn	1364
70	GCA TAC ACG CCA GTT GCA GTA ACT AAT TAT GAA ACA AAA CTA TTA TCA Ala Tyr Thr Pro Val Ala Val Thr Asn Tyr Glu Thr Lys Leu Leu Ser	1412
75	ACT TCA TCT TTT TGG AAA TTT ATT TCT AGG GAT CCA GGA TGG GTA GAG Thr Ser Ser Phe Trp Lys Phe Ile Ser Arg Asp Pro Gly Trp Val Glu	1460
80	TAAAAGCTT	1469

(2) INFORMATION FOR SEQ ID NO:68:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 451 amino acids

(B) TYPE: amino acid  
(D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:68:

Met Gly His His His His His His His His His Ser Ser Gly His  
1 5 10 15  
Ile Glu Gly Arg His Met Ala Ser Met Ala Leu Leu Lys Asp Ile Ile  
20 25 30  
Asn Glu Tyr Phe Asn Ser Ile Asn Asp Ser Lys Ile Leu Ser Leu Gln  
35 40 45  
Asn Lys Lys Asn Ala Leu Val Asp Thr Ser Gly Tyr Asn Ala Glu Val  
50 55 60  
Arg Val Gly Asp Asn Val Gln Leu Asn Thr Ile Tyr Thr Asn Asp Phe  
65 70 75 80  
Lys Leu Ser Ser Ser Gly Asp Lys Ile Ile Val Asn Leu Asn Asn Asn  
85 90 95  
Ile Leu Tyr Ser Ala Ile Tyr Glu Asn Ser Ser Val Ser Phe Trp Ile  
100 105 110  
Lys Ile Ser Lys Asp Leu Thr Asn Ser His Asn Glu Tyr Thr Ile Ile  
115 120 125  
Asn Ser Ile Glu Gln Asn Ser Gly Trp Lys Leu Cys Ile Arg Asn Gly  
130 135 140  
Asn Ile Glu Trp Ile Leu Gln Asp Val Asn Arg Lys Tyr Lys Ser Leu  
145 150 155 160  
Ile Phe Asp Tyr Ser Glu Ser Leu Ser His Thr Gly Tyr Thr Asn Lys  
165 170 175  
Trp Phe Phe Val Thr Ile Thr Asn Asn Ile Met Gly Tyr Met Lys Leu  
180 185 190  
Tyr Ile Asn Gly Glu Leu Lys Gln Ser Gln Lys Ile Glu Asp Leu Asp  
195 200 205  
Glu Val Lys Leu Asp Lys Thr Ile Val Phe Gly Ile Asp Glu Asn Ile  
210 215 220  
Asp Glu Asn Gln Met Leu Trp Ile Arg Asp Phe Asn Ile Phe Ser Lys  
225 230 235 240  
Glu Leu Ser Asn Glu Asp Ile Asn Ile Val Tyr Glu Gly Gln Ile Leu  
245 250 255  
Arg Asn Val Ile Lys Asp Tyr Trp Gly Asn Pro Leu Lys Phe Asp Thr  
260 265 270  
Glu Tyr Tyr Ile Ile Asn Asp Asn Tyr Ile Asp Arg Tyr Ile Ala Pro  
275 280 285  
Glu Ser Asn Val Leu Val Leu Val Arg Tyr Pro Asp Arg Ser Lys Leu  
290 295 300  
Tyr Thr Gly Asn Pro Ile Thr Ile Lys Ser Val Ser Asp Lys Asn Pro  
305 310 315 320  
Tyr Ser Arg Ile Leu Asn Gly Asp Asn Ile Ile Leu His Met Leu Tyr  
325 330 335



Asn Ser Arg Lys Tyr Met Ile Ile Arg Asp Thr Asp Thr Ile Tyr Ala  
 340 345 350  
 5 Thr Gln Gly Gly Glu Cys Ser Gln Asn Cys Val Tyr Ala Leu Lys Leu  
 355 360 365  
 Gln Ser Asn Leu Gly Asn Tyr Gly Ile Gly Ile Phe Ser Ile Lys Asn  
 370 375 380  
 10 Ile Val Ser Lys Asn Lys Tyr Cys Ser Gln Ile Phe Ser Ser Phe Arg  
 385 390 395 400  
 Glu Asn Thr Met Leu Leu Ala Asp Ile Tyr Lys Pro Trp Arg Phe Ser  
 405 410 415  
 15 Phe Lys Asn Ala Tyr Thr Pro Val Ala Val Thr Asn Tyr Glu Thr Lys  
 420 425 430  
 20 Leu Leu Ser Thr Ser Ser Phe Trp Lys Phe Ile Ser Arg Asp Pro Gly  
 435 440 445  
 Trp Val Glu  
 450  
 25 (2) INFORMATION FOR SEQ ID NO:69:  
 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 32 base pairs  
 (B) TYPE: nucleic acid  
 30 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear  
 (ii) MOLECULE TYPE: other nucleic acid  
 (A) DESCRIPTION: /desc = "DNA"  
 35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:69:  
 GCAAGCTTTT ACTCTACCCA TCCTGGATCC CT 32  
 40 (2) INFORMATION FOR SEQ ID NO:70:  
 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 3825 base pairs  
 (B) TYPE: nucleic acid  
 45 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear  
 (ii) MOLECULE TYPE: DNA (genomic)  
 50 (ix) FEATURE:  
 (A) NAME/KEY: CDS  
 (B) LOCATION: 1..3822  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:70:  
 55 ATG CCA GTT GCA ATA AAT AGT TTT AAT TAT AAT GAC CCT GTT AAT GAT 48  
 Met Pro Val Ala Ile Asn Ser Phe Asn Tyr Asn Asp Pro Val Asn Asp  
 1 5 10 15  
 60 GAT ACA ATT TTA TAC ATG CAG ATA CCA TAT GAA GAA AAA AGT AAA AAA 96  
 Asp Thr Ile Leu Tyr Met Gln Ile Pro Tyr Glu Glu Lys Ser Lys Lys  
 20 25 30  
 65 TAT TAT AAA GCT TTT GAG ATT ATG CGT AAT GTT TGG ATA ATT CCT GAG 144  
 Tyr Tyr Lys Ala Phe Glu Ile Met Arg Asn Val Trp Ile Ile Pro Glu  
 35 40 45  
 70 AGA AAT ACA ATA GGA ACG AAT CCT AGT GAT TTT GAT CCA CCG GCT TCA 192  
 Arg Asn Thr Ile Gly Thr Asn Pro Ser Asp Phe Asp Pro Pro Ala Ser  
 50 55 60

	TTA AAG AAC GGA AGC AGT GCT TAT TAT GAT CCT AAT TAT TTA ACC ACT Leu Lys Asn Gly Ser Ser Ala Tyr Tyr Asp Pro Asn Tyr Leu Thr Thr 65 70 75 80	240
5	GAT GCT GAA AAA GAT AGA TAT TTA AAA ACA ACG ATA AAA TTA TTT AAG Asp Ala Glu Lys Asp Arg Tyr Leu Lys Thr Thr Ile Lys Leu Phe Lys 85 90 95	288
10	AGA ATT AAT AGT AAT CCT GCA GGG AAA GTT TTG TTA CAA GAA ATA TCA Arg Ile Asn Ser Asn Pro Ala Gly Lys Val Leu Leu Gln Glu Ile Ser 100 105 110	336
15	TAT GCT AAA CCA TAT TTA GGA AAT GAC CAC ACG CCA ATT GAT GAA TTC Tyr Ala Lys Pro Tyr Leu Gly Asn Asp His Thr Pro Ile Asp Glu Phe 115 120 125	384
20	TCT CCA GTT ACT AGA ACT ACA AGT GTT AAT ATA AAA TTA TCA ACT AAT Ser Pro Val Thr Arg Thr Thr Ser Val Asn Ile Lys Leu Ser Thr Asn 130 135 140	432
	GTT GAA AGT TCA ATG TTA TTG AAT CTT CTT GTA TTG GGA GCA GGA CCT Val Glu Ser Ser Met Leu Leu Asn Leu Leu Val Leu Gly Ala Gly Pro 145 150 155 160	480
25	GAT ATA TTT GAA AGT TGT TGT TAC CCC GTT AGA AAA CTA ATA GAT CCA Asp Ile Phe Glu Ser Cys Cys Tyr Pro Val Arg Lys Leu Ile Asp Pro 165 170 175	528
30	GAT GTA GTT TAT GAT CCA AGT AAT TAT GGT TTT GGA TCA ATT AAT ATC Asp Val Val Tyr Asp Pro Ser Asn Tyr Gly Phe Gly Ser Ile Asn Ile 180 185 190	576
35	GTG ACA TTT TCA CCT GAG TAT GAA TAT ACT TTT AAT GAT ATT AGT GGA Val Thr Phe Ser Pro Glu Tyr Glu Tyr Thr Phe Asn Asp Ile Ser Gly 195 200 205	624
40	GGG CAT AAT AGT AGT ACA GAA TCA TTT ATT GCA GAT CCT GCA ATT TCA Gly His Asn Ser Ser Thr Glu Ser Phe Ile Ala Asp Pro Ala Ile Ser 210 215 220	672
	CTA GCT CAT GAA TTG ATA CAT GCA CTG CAT GGA TTA TAC GGG GCT AGG Leu Ala His Glu Leu Ile His Ala Leu His Gly Leu Tyr Gly Ala Arg 225 230 235 240	720
45	GGA GTT ACT TAT GAA GAG ACT ATA GAA GTA AAG CAA GCA CCT CTT ATG Gly Val Thr Tyr Glu Glu Thr Ile Glu Val Lys Gln Ala Pro Leu Met 245 250 255	768
50	ATA GCC GAA AAA CCC ATA AGG CTA GAA GAA TTT TTA ACC TTT GGA GGT Ile Ala Glu Lys Pro Ile Arg Leu Glu Glu Phe Leu Thr Phe Gly Gly 260 265 270	816
55	CAG GAT TTA AAT ATT ATT ACT AGT GCT ATG AAG GAA AAA ATA TAT AAC Gln Asp Leu Asn Ile Ile Thr Ser Ala Met Lys Glu Lys Ile Tyr Asn 275 280 285	864
60	AAT CTT TTA GCT AAC TAT GAA AAA ATA GCT ACT AGA CTT AGT GAA GTT Asn Leu Leu Ala Asn Tyr Glu Lys Ile Ala Thr Arg Leu Ser Glu Val 290 295 300	912
	AAT AGT GCT CCT CCT GAA TAT GAT ATT AAT GAA TAT AAA GAT TAT TTT Asn Ser Ala Pro Pro Glu Tyr Asp Ile Asn Glu Tyr Lys Asp Tyr Phe 305 310 315 320	960
65	CAA TGG AAG TAT GGG CTA GAT AAA AAT GCT GAT GGA AGT TAT ACT GTA Gln Trp Lys Tyr GGG Leu Asp Lys Asn Ala Asp Gly Ser Tyr Thr Val 325 330 335	1008
70	AAT GAA AAT AAA TTT AAT GAA ATT TAT AAA AAA TTA TAT AGT TTT ACA Asn Glu Asn Lys Phe Asn Glu Ile Tyr Lys Lys Leu Tyr Ser Phe Thr	1056

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	GGT CTT GCT TTG AAT ATA ATT ATT GAG GCA GAA AAA GGA AAT TTT GAG Gly Leu Ala Leu Asn Ile Ile Ile Glu Ala Glu Lys Gly Asn Phe Glu 625 630 635 640	1920
5	GAG GCA TTT GAA TTA TTA GGA GTG GGT ATT TTA TTA GAA TTT GTG CCA Glu Ala Phe Glu Leu Leu Gly Val Gly Ile Leu Leu Glu Phe Val Pro 645 650 655	1968
10	GAA CTT ACA ATT CCT GTA ATT TTA GTG TTT ACG ATA AAA TCC TAT ATA Glu Leu Thr Ile Pro Val Ile Leu Val Phe Thr Ile Lys Ser Tyr Ile 660 665 670	2016
15	GAT TCA TAT GAG AAT AAA AAT AAA GCA ATT AAA GCA ATA AAT AAT TCA Asp Ser Tyr Glu Asn Lys Asn Lys Ala Ile Lys Ala Ile Asn Asn Ser 675 680 685	2064
20	TTA ATC GAA AGA GAA GCA AAG TGG AAA GAA ATA TAT AGT TGG ATA GTA Leu Ile Glu Arg Glu Ala Lys Trp Lys Glu Ile Tyr Ser Trp Ile Val 690 695 700	2112
	TCA AAT TGG CTT ACT AGA ATT AAT ACT CAA TTT AAT AAA AGA AAA GAG Ser Asn Trp Leu Thr Arg Ile Asn Thr Gln Phe Asn Lys Arg Lys Glu 705 710 715 720	2160
25	CAA ATG TAT CAG GCT TTA CAA AAT CAA GTA GAT GCA ATA AAA ACA GCA Gln Met Tyr Gln Ala Leu Gln Asn Gln Val Asp Ala Ile Lys Thr Ala 725 730 735	2208
30	ATA GAA TAT AAA TAT AAT AAT TAT ACT TCA GAT GAG AAA AAT AGA CTT Ile Glu Tyr Lys Tyr Asn Asn Tyr Thr Ser Asp Glu Lys Asn Arg Leu 740 745 750	2256
35	GAA TCT GAA TAT AAT ATC AAT AAT ATA GAA GAA GAA TTG AAT AAA AAA Glu Ser Glu Tyr Asn Ile Asn Asn Ile Glu Glu Glu Leu Asn Lys Lys 755 760 765	2304
40	GTT TCT TTA GCA ATG AAA AAT ATA GAA AGA TTT ATG ACA GAA AGT TCT Val Ser Leu Ala Met Lys Asn Ile Glu Arg Phe Met Thr Glu Ser Ser 770 775 780	2352
	ATA TCT TAT TTA ATG AAA TTA ATA AAT GAA GCC AAA GTT GGT AAA TTA Ile Ser Tyr Leu Met Lys Leu Ile Asn Glu Ala Lys Val Gly Lys Leu 785 790 795 800	2400
45	AAA AAA TAT GAT AAC CAT GTT AAG AGC GAT TTA TTA AAC TAT ATT CTC Lys Lys Tyr Asp Asn His Val Lys Ser Asp Leu Leu Asn Tyr Ile Leu 805 810 815	2448
50	GAC CAT AGA TCA ATC TTA GGA GAG CAG ACA AAT GAA TTA AGT GAT TTG Asp His Arg Ser Ile Leu Gly Glu Gln Thr Asn Glu Leu Ser Asp Leu 820 825 830	2496
55	GTG ACT AGT ACT TTG AAT AGT AGT ATT CCA TTT GAA CTT TCT TCA TAT Val Thr Ser Thr Leu Asn Ser Ser Ile Pro Phe Glu Leu Ser Ser Tyr 835 840 845	2544
	ACT AAT GAT AAA ATT CTA ATT ATA TAT TTT AAT AGA TTA TAT AAA AAA Thr Asn Asp Lys Ile Leu Ile Ile Tyr Phe Asn Arg Leu Tyr Lys Lys 850 855 860	2592
60	ATT AAA GAT AGT TCT ATT TTA GAT ATG CGA TAT GAA AAT AAT AAA TTT Ile Lys Asp Ser Ser Ile Leu Asp Met Arg Tyr Glu Asn Asn Lys Phe 865 870 875 880	2640

	ATA GAT ATC TCT GGA TAT GGT TCA AAT ATA AGC ATT AAT GGA AAC GTA Ile Asp Ile Ser Gly Tyr Gly Ser Asn Ile Ser Ile Asn Gly Asn Val 885 890 895	2688
5	TAT ATT TAT TCA ACA AAT AGA AAT CAA TTT GGA ATA TAT AAT AGT AGG Tyr Ile Tyr Ser Thr Asn Arg Asn Gln Phe Gly Ile Tyr Asn Ser Arg 900 905 910	2736
10	CTT AGT GAA GTT AAT ATA GCT CAA AAT AAT GAT ATT ATA TAC AAT AGT Leu Ser Glu Val Asn Ile Ala Gln Asn Asn Asp Ile Ile Tyr Asn Ser 915 920 925	2784
15	AGA TAT CAA AAT TTT AGT ATT AGT TTC TGG GTA AGG ATT CCT AAA CAC Arg Tyr Gln Asn Phe Ser Ile Ser Phe Trp Val Arg Ile Pro Lys His 930 935 940	2832
20	TAC AAA CCT ATG AAT CAT AAT CGG GAA TAC ACT ATA ATA AAT TGT ATG Tyr Lys Pro Met Asn His Asn Arg Glu Tyr Thr Ile Ile Asn Cys Met 945 950 955 960	2880
	GGG AAT AAT AAT TCG GGA TGG AAA ATA TCA CTT AGA ACT GTT AGA GAT Gly Asn Asn Asn Ser Gly Trp Lys Ile Ser Leu Arg Thr Val Arg Asp 965 970 975	2928
25	TGT GAA ATA ATT TGG ACT TTA CAA GAT ACT TCT GGA AAT AAG GAA AAT Cys Glu Ile Ile Trp Thr Leu Gln Asp Thr Ser Gly Asn Lys Glu Asn 980 985 990	2976
30	TTA ATT TTT AGG TAT GAA GAA CTT AAT AGG ATA TCT AAT TAT ATA AAT Leu Ile Phe Arg Tyr Glu Glu Leu Asn Arg Ile Ser Asn Tyr Ile Asn 995 1000 1005	3024
35	AAA TGG ATT TTT GTA ACT ATT ACT AAT AAT AGA TTA GGC AAT TCT AGA Lys Trp Ile Phe Val Thr Ile Thr Asn Asn Arg Leu Gly Asn Ser Arg 1010 1015 1020	3072
40	ATT TAC ATC AAT GGA AAT TTA ATA GTT GAA AAA TCA ATT TCG AAT TTA Ile Tyr Ile Asn Gly Asn Leu Ile Val Glu Lys Ser Ile Ser Asn Leu 1025 1030 1035 1040	3120
	GGT GAT ATT CAT GTT AGT GAT AAT ATA TTA TTT AAA ATT GTT GGT TGT Gly Asp Ile His Val Ser Asp Asn Ile Leu Phe Lys Ile Val Gly Cys 1045 1050 1055	3168
45	GAT GAT GAA ACG TAT GTT GGT ATA AGA TAT TTT AAA GTT TTT AAT ACG Asp Asp Glu Thr Tyr Val Gly Ile Arg Tyr Phe Lys Val Phe Asn Thr 1060 1065 1070	3216
50	GAA TTA GAT AAA ACA GAA ATT GAG ACT TTA TAT AGT AAT GAG CCA GAT Glu Leu Asp Lys Thr Glu Ile Glu Thr Leu Tyr Ser Asn Glu Pro Asp 1075 1080 1085	3264
55	CCA AGT ATC TTA AAA AAC TAT TGG GGA AAT TAT TTG CTA TAT AAT AAA Pro Ser Ile Leu Lys Asn Tyr Trp Gly Asn Tyr Leu Leu Tyr Asn Lys 1090 1095 1100	3312
60	AAA TAT TAT TTA TTC AAT TTA CTA AGA AAA GAT AAG TAT ATT ACT CTG Lys Tyr Tyr Leu Phe Asn Leu Leu Arg Lys Asp Lys Tyr Ile Thr Leu 1105 1110 1115 1120	3360
	AAT TCA GGC ATT TTA AAT ATT AAT CAA CAA AGA GGT GTT ACT GAA GGC Asn Ser Gly Ile Leu Asn Ile Asn Gln Gln Arg Gly Val Thr Glu Gly 1125 1130 1135	3408
65	TCT GTT TTT TTG AAC TAT AAA TTA TAT GAA GGA GTA GAA GTC ATT ATA Ser Val Phe Leu Asn Tyr Lys Leu Tyr Glu Gly Val Glu Val Ile Ile 1140 1145 1150	3456
70	AGA AAA AAT GGT CCT ATA GAT ATA TCT AAT ACA GAT AAT TTT GTT AGA Arg Lys Asn Gly Pro Ile Asp Ile Ser Asn Thr Asp Asn Phe Val Arg	3504

	1155	1160	1165	
5	AAA AAC GAT CTA GCA TAC ATT AAT GTA GTA GAT CGT GGT GTA GAA TAT Lys Asn Asp Leu Ala Tyr Ile Asn Val Val Asp Arg Gly Val Glu Tyr 1170 1175 1180	3552		
10	CGG TTA TAT GCT GAT ACA AAA TCA GAG AAA GAG AAA ATA ATA AGA ACA Arg Leu Tyr Ala Asp Thr Lys Ser Glu Lys Glu Lys Ile Ile Arg Thr 1185 1190 1195 1200	3600		
15	TCT AAT CTA AAC GAT AGC TTA GGT CAA ATT ATA GTT ATG GAT TCA ATA Ser Asn Leu Asn Asp Ser Leu Gly Gln Ile Ile Val Met Asp Ser Ile 1205 1210 1215	3648		
20	GGA AAT AAT TGC ACA ATG AAT TTT CAA AAC AAT AAT GGG AGC AAT ATA Gly Asn Asn Cys Thr Met Asn Phe Gln Asn Asn Asn Gly Ser Asn Ile 1220 1225 1230	3696		
25	GGA TTA CTA GGT TTT CAT TCA AAT AAT TTG GTT GCT AGT AGT TGG TAT Gly Leu Leu Gly Phe His Ser Asn Asn Leu Val Ala Ser Ser Trp Tyr 1235 1240 1245	3744		
30	TAT AAC AAT ATA CGA AGA AAT ACT AGC AGT AAT GGA TGC TTT TGG AGT Tyr Asn Asn Ile Arg Arg Asn Thr Ser Ser Asn Gly Cys Phe Trp Ser 1250 1255 1260	3792		
35	TCT ATT TCT AAA GAG AAT GGA TGG AAA GAA TGA Ser Ile Ser Lys Glu Asn Gly Trp Lys Glu 1265 1270	3825		
	(2) INFORMATION FOR SEQ ID NO:71:			
	(i) SEQUENCE CHARACTERISTICS:			
	(A) LENGTH: 1274 amino acids			
	(B) TYPE: amino acid			
	(D) TOPOLOGY: linear			
	(ii) MOLECULE TYPE: protein			
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:71:			
40	Met Pro Val Ala Ile Asn Ser Phe Asn Tyr Asn Asp Pro Val Asn Asp 1 5 10 15			
45	Asp Thr Ile Leu Tyr Met Gln Ile Pro Tyr Glu Glu Lys Ser Lys Lys 20 25 30			
50	Tyr Tyr Lys Ala Phe Glu Ile Met Arg Asn Val Trp Ile Ile Pro Glu 35 40 45			
55	Arg Asn Thr Ile Gly Thr Asn Pro Ser Asp Phe Asp Pro Pro Ala Ser 50 55 60			
60	Leu Lys Asn Gly Ser Ser Ala Tyr Tyr Asp Pro Asn Tyr Leu Thr Thr 65 70 75 80			
65	Asp Ala Glu Lys Asp Arg Tyr Leu Lys Thr Thr Ile Lys Leu Phe Lys 85 90 95			
70	Arg Ile Asn Ser Asn Pro Ala Gly Lys Val Leu Leu Gln Glu Ile Ser 100 105 110			
	Tyr Ala Lys Pro Tyr Leu Gly Asn Asp His Thr Pro Ile Asp Glu Phe 115 120 125			
	Ser Pro Val Thr Arg Thr Thr Ser Val Asn Ile Lys Leu Ser Thr Asn 130 135 140			
	Val Glu Ser Ser Met Leu Leu Asn Leu Leu Val Leu Gly Ala Gly Pro 145 150 155 160			

Asp Ile Phe Glu Ser Cys Cys Tyr Pro Val Arg Lys Leu Ile Asp Pro  
 165 170 175  
 5 Asp Val Val Tyr Asp Pro Ser Asn Tyr Gly Phe Gly Ser Ile Asn Ile  
 180 185 190  
 Val Thr Phe Ser Pro Glu Tyr Glu Tyr Thr Phe Asn Asp Ile Ser Gly  
 195 200 205  
 10 Gly His Asn Ser Ser Thr Glu Ser Phe Ile Ala Asp Pro Ala Ile Ser  
 210 215 220  
 Leu Ala His Glu Leu Ile His Ala Leu His Gly Leu Tyr Gly Ala Arg  
 225 230 235 240  
 15 Gly Val Thr Tyr Glu Glu Thr Ile Glu Val Lys Gln Ala Pro Leu Met  
 245 250 255  
 20 Ile Ala Glu Lys Pro Ile Arg Leu Glu Glu Phe Leu Thr Phe Gly Gly  
 260 265 270  
 Gln Asp Leu Asn Ile Ile Thr Ser Ala Met Lys Glu Lys Ile Tyr Asn  
 275 280 285  
 25 Asn Leu Leu Ala Asn Tyr Glu Lys Ile Ala Thr Arg Leu Ser Glu Val  
 290 295 300  
 Asn Ser Ala Pro Pro Glu Tyr Asp Ile Asn Glu Tyr Lys Asp Tyr Phe  
 305 310 315 320  
 30 Gln Trp Lys Tyr Gly Leu Asp Lys Asn Ala Asp Gly Ser Tyr Thr Val  
 325 330 335  
 35 Asn Glu Asn Lys Phe Asn Glu Ile Tyr Lys Lys Leu Tyr Ser Phe Thr  
 340 345 350  
 Glu Ser Asp Leu Ala Asn Lys Phe Lys Val Lys Cys Arg Asn Thr Tyr  
 355 360 365  
 40 Phe Ile Lys Tyr Glu Phe Leu Lys Val Pro Asn Leu Leu Asp Asp Asp  
 370 375 380  
 Ile Tyr Thr Val Ser Glu Gly Phe Asn Ile Gly Asn Leu Ala Val Asn  
 385 390 395 400  
 45 Asn Arg Gly Gln Ser Ile Lys Leu Asn Pro Lys Ile Ile Asp Ser Ile  
 405 410 415  
 50 Pro Asp Lys Gly Leu Val Glu Lys Ile Val Lys Phe Cys Lys Ser Val  
 420 425 430  
 Ile Pro Arg Lys Gly Thr Lys Ala Pro Pro Arg Leu Cys Ile Arg Val  
 435 440 445  
 55 Asn Asn Ser Glu Leu Phe Phe Val Ala Ser Glu Ser Ser Tyr Asn Glu  
 450 455 460  
 Asn Asp Ile Asn Thr Pro Lys Glu Ile Asp Asp Thr Thr Asn Leu Asn  
 465 470 475 480  
 60 Asn Asn Tyr Arg Asn Asn Leu Asp Glu Val Ile Leu Asp Tyr Asn Ser  
 485 490 495  
 Gln Thr Ile Pro Gln Ile Ser Asn Arg Thr Leu Asn Thr Leu Val Gln  
 500 505 510  
 65 Asp Asn Ser Tyr Val Pro Arg Tyr Asp Ser Asn Gly Thr Ser Glu Ile  
 515 520 525  
 70 Glu Glu Tyr Asp Val Val Asp Phe Asn Val Phe Phe Tyr Leu His Ala

	530	535	540
5	Gln Lys Val Pro Glu Gly Glu Thr Asn Ile Ser Leu Thr Ser Ser Ile 545 550 555 560		
	Asp Thr Ala Leu Leu Glu Glu Ser Lys Asp Ile Phe Phe Ser Ser Glu 565 570 575		
10	Phe Ile Asp Thr Ile Asn Lys Pro Val Asn Ala Ala Leu Phe Ile Asp 580 585 590		
	Trp Ile Ser Lys Val Ile Arg Asp Phe Thr Thr Glu Ala Thr Gln Lys 595 600 605		
15	Ser Thr Val Asp Lys Ile Ala Asp Ile Ser Leu Ile Val Pro Tyr Val 610 615 620		
	Gly Leu Ala Leu Asn Ile Ile Ile Glu Ala Glu Lys Gly Asn Phe Glu 625 630 635 640		
20	Glu Ala Phe Glu Leu Leu Gly Val Gly Ile Leu Leu Glu Phe Val Pro 645 650 655		
	Glu Leu Thr Ile Pro Val Ile Leu Val Phe Thr Ile Lys Ser Tyr Ile 660 665 670		
25	Asp Ser Tyr Glu Asn Lys Asn Lys Ala Ile Lys Ala Ile Asn Asn Ser 675 680 685		
30	Leu Ile Glu Arg Glu Ala Lys Trp Lys Glu Ile Tyr Ser Trp Ile Val 690 695 700		
	Ser Asn Trp Leu Thr Arg Ile Asn Thr Gln Phe Asn Lys Arg Lys Glu 705 710 715 720		
35	Gln Met Tyr Gln Ala Leu Gln Asn Gln Val Asp Ala Ile Lys Thr Ala 725 730 735		
	Ile Glu Tyr Lys Tyr Asn Asn Tyr Thr Ser Asp Glu Lys Asn Arg Leu 740 745 750		
40	Glu Ser Glu Tyr Asn Ile Asn Asn Ile Glu Glu Glu Leu Asn Lys Lys 755 760 765		
45	Val Ser Leu Ala Met Lys Asn Ile Glu Arg Phe Met Thr Glu Ser Ser 770 775 780		
	Ile Ser Tyr Leu Met Lys Leu Ile Asn Glu Ala Lys Val Gly Lys Leu 785 790 795 800		
50	Lys Lys Tyr Asp Asn His Val Lys Ser Asp Leu Leu Asn Tyr Ile Leu 805 810 815		
55	Asp His Arg Ser Ile Leu Gly Glu Gln Thr Asn Glu Leu Ser Asp Leu 820 825 830		
	Val Thr Ser Thr Leu Asn Ser Ser Ile Pro Phe Glu Leu Ser Ser Tyr 835 840 845		
60	Thr Asn Asp Lys Ile Leu Ile Ile Tyr Phe Asn Arg Leu Tyr Lys Lys 850 855 860		
	Ile Lys Asp Ser Ser Ile Leu Asp Met Arg Tyr Glu Asn Asn Lys Phe 865 870 875 880		
65	Ile Asp Ile Ser Gly Tyr Gly Ser Asn Ile Ser Ile Asn Gly Asn Val 885 890 895		
70	Tyr Ile Tyr Ser Thr Asn Arg Asn Gln Phe Gly Ile Tyr Asn Ser Arg 900 905 910		



Leu Ser Glu Val Asn Ile Ala Gln Asn Asn Asp Ile Ile Tyr Asn Ser  
 915 920 925  
 5 Arg Tyr Gln Asn Phe Ser Ile Ser Phe Trp Val Arg Ile Pro Lys His  
 930 935 940  
 Tyr Lys Pro Met Asn His Asn Arg Glu Tyr Thr Ile Ile Asn Cys Met  
 945 950 955 960  
 10 Gly Asn Asn Asn Ser Gly Trp Lys Ile Ser Leu Arg Thr Val Arg Asp  
 965 970 975  
 Cys Glu Ile Ile Trp Thr Leu Gln Asp Thr Ser Gly Asn Lys Glu Asn  
 980 985 990  
 15 Leu Ile Phe Arg Tyr Glu Glu Leu Asn Arg Ile Ser Asn Tyr Ile Asn  
 995 1000 1005  
 Lys Trp Ile Phe Val Thr Ile Thr Asn Asn Arg Leu Gly Asn Ser Arg  
 1010 1015 1020  
 Ile Tyr Ile Asn Gly Asn Leu Ile Val Glu Lys Ser Ile Ser Asn Leu  
 1025 1030 1035 1040  
 25 Gly Asp Ile His Val Ser Asp Asn Ile Leu Phe Lys Ile Val Gly Cys  
 1045 1050 1055  
 Asp Asp Glu Thr Tyr Val Gly Ile Arg Tyr Phe Lys Val Phe Asn Thr  
 1060 1065 1070  
 30 Glu Leu Asp Lys Thr Glu Ile Glu Thr Leu Tyr Ser Asn Glu Pro Asp  
 1075 1080 1085  
 Pro Ser Ile Leu Lys Asn Tyr Trp Gly Asn Tyr Leu Leu Tyr Asn Lys  
 1090 1095 1100  
 Lys Tyr Tyr Leu Phe Asn Leu Leu Arg Lys Asp Lys Tyr Ile Thr Leu  
 1105 1110 1115 1120  
 40 Asn Ser Gly Ile Leu Asn Ile Asn Gln Gln Arg Gly Val Thr Glu Gly  
 1125 1130 1135  
 Ser Val Phe Leu Asn Tyr Lys Leu Tyr Glu Gly Val Glu Val Ile Ile  
 1140 1145 1150  
 45 Arg Lys Asn Gly Pro Ile Asp Ile Ser Asn Thr Asp Asn Phe Val Arg  
 1155 1160 1165  
 Lys Asn Asp Leu Ala Tyr Ile Asn Val Val Asp Arg Gly Val Glu Tyr  
 1170 1175 1180  
 Arg Leu Tyr Ala Asp Thr Lys Ser Glu Lys Glu Lys Ile Ile Arg Thr  
 1185 1190 1195 1200  
 55 Ser Asn Leu Asn Asp Ser Leu Gly Gln Ile Ile Val Met Asp Ser Ile  
 1205 1210 1215  
 Gly Asn Asn Cys Thr Met Asn Phe Gln Asn Asn Asn Gly Ser Asn Ile  
 1220 1225 1230  
 60 Gly Leu Leu Gly Phe His Ser Asn Asn Leu Val Ala Ser Ser Trp Tyr  
 1235 1240 1245  
 Tyr Asn Asn Ile Arg Arg Asn Thr Ser Ser Asn Gly Cys Phe Trp Ser  
 1250 1255 1260  
 Ser Ile Ser Lys Glu Asn Gly Trp Lys Glu  
 1265 1270  
 70 (2) INFORMATION FOR SEQ ID NO:72:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 1460 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:  
 (A) NAME/KEY: CDS  
 (B) LOCATION: 108..1451

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:72:

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15 AGATCTCGAT CCCGCGAAAT TAATACGACT CACTATAGGG GAATTGTGAG CGGATAACAA      60
   TTCCCTCTA GAAATAATTT TGTTTAACTT TAAGAAGGAG ATATACC ATG GGC CAT      116
                                     Met Gly His
                                     1
20 CAT CAT CAT CAT CAT CAT CAT CAT CAC AGC AGC GGC CAT ATC GAA GGT      164
   His His His His His His His His His Ser Ser Gly His Ile Glu Gly
       5                      10                      15
25 CGT CAT ATG GCT AGC ATG GCT ATT CTA ATT ATA TAT TTT AAT AGA TTA      212
   Arg His Met Ala Ser Met Ala Ile Leu Ile Ile Tyr Phe Asn Arg Leu
       20                      25                      30                      35
30 TAT AAA AAA ATT AAA GAT AGT TCT ATT TTA GAT ATG CGA TAT GAA AAT      260
   Tyr Lys Lys Ile Lys Asp Ser Ser Ile Leu Asp Met Arg Tyr Glu Asn
                                     40                      45                      50
35 AAT AAA TTT ATA GAT ATC TCT GGA TAT GGT TCA AAT ATA AGC ATT AAT      308
   Asn Lys Phe Ile Asp Ile Ser Gly Tyr Gly Ser Asn Ile Ser Ile Asn
                                     55                      60                      65
   GGA AAC GTA TAT ATT TAT TCA ACA AAT AGA AAT CAA TTT GGA ATA TAT      356
   Gly Asn Val Tyr Ile Tyr Ser Thr Asn Arg Asn Gln Phe Gly Ile Tyr
                                     70                      75                      80
40 AAT AGT AGG CTT AGT GAA GTT AAT ATA GCT CAA AAT AAT GAT ATT ATA      404
   Asn Ser Arg Leu Ser Glu Val Asn Ile Ala Gln Asn Asn Asp Ile Ile
                                     85                      90                      95
45 TAC AAT AGT AGA TAT CAA AAT TTT AGT ATT AGT TTC TGG GTA AGG ATT      452
   Tyr Asn Ser Arg Tyr Gln Asn Phe Ser Ile Ser Phe Trp Val Arg Ile
       100                      105                      110                      115
50 CCT AAA CAC TAC AAA CCT ATG AAT CAT AAT CGG GAA TAC ACT ATA ATA      500
   Pro Lys His Tyr Lys Pro Met Asn His Asn Arg Glu Tyr Thr Ile Ile
                                     120                      125                      130
55 AAT TGT ATG GGG AAT AAT AAT TCG GGA TGG AAA ATA TCA CTT AGA ACT      548
   Asn Cys Met Gly Asn Asn Asn Ser Gly Trp Lys Ile Ser Leu Arg Thr
                                     135                      140                      145
   GTT AGA GAT TGT GAA ATA ATT TGG ACT TTA CAA GAT ACT TCT GGA AAT      596
   Val Arg Asp Cys Glu Ile Ile Trp Thr Leu Gln Asp Thr Ser Gly Asn
       150                      155                      160
60 AAG GAA AAT TTA ATT TTT AGG TAT GAA GAA CTT AAT AGG ATA TCT AAT      644
   Lys Glu Asn Leu Ile Phe Arg Tyr Glu Glu Leu Asn Arg Ile Ser Asn
       165                      170                      175
65 TAT ATA AAT AAA TGG ATT TTT GTA ACT ATT ACT AAT AAT AGA TTA GGC      692
   Tyr Ile Asn Lys Trp Ile Phe Val Thr Ile Thr Asn Asn Arg Leu Gly
       180                      185                      190                      195
70 AAT TCT AGA ATT TAC ATC AAT GGA AAT TTA ATA GTT GAA AAA TCA ATT      740
   Asn Ser Arg Ile Tyr Ile Asn Gly Asn Leu Ile Val Glu Lys Ser Ile

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	200	205	210	
5	TCG AAT TTA GGT GAT ATT CAT GTT AGT GAT AAT ATA TTA TTT AAA ATT Ser Asn Leu Gly Asp Ile His Val Ser Asp Asn Ile Leu Phe Lys Ile 215 220 225			788
10	GTT GGT TGT GAT GAT GAA ACG TAT GTT GGT ATA AGA TAT TTT AAA GTT Val Gly Cys Asp Asp Glu Thr Tyr Val Gly Ile Arg Tyr Phe Lys Val 230 235 240			836
15	TTT AAT ACG GAA TTA GAT AAA ACA GAA ATT GAG ACT TTA TAT AGT AAT Phe Asn Thr Glu Leu Asp Lys Thr Glu Ile Glu Thr Leu Tyr Ser Asn 245 250 255			884
20	GAG CCA GAT CCA AGT ATC TTA AAA AAC TAT TGG GGA AAT TAT TTG CTA Glu Pro Asp Pro Ser Ile Leu Lys Asn Tyr Trp Gly Asn Tyr Leu Leu 260 265 270 275			932
25	TAT AAT AAA AAA TAT TAT TTA TTC AAT TTA CTA AGA AAA GAT AAG TAT Tyr Asn Lys Lys Tyr Tyr Leu Phe Asn Leu Leu Arg Lys Asp Lys Tyr 280 285 290			980
30	ATT ACT CTG AAT TCA GGC ATT TTA AAT ATT AAT CAA CAA AGA GGT GTT Ile Thr Leu Asn Ser Gly Ile Leu Asn Ile Asn Gln Gln Arg Gly Val 295 300 305			1028
35	ACT GAA GGC TCT GTT TTT TTG AAC TAT AAA TTA TAT GAA GGA GTA GAA Thr Glu Gly Ser Val Phe Leu Asn Tyr Lys Leu Tyr Glu Gly Val Glu 310 315 320			1076
40	GTC ATT ATA AGA AAA AAT GGT CCT ATA GAT ATA TCT AAT ACA GAT AAT Val Ile Ile Arg Lys Asn Gly Pro Ile Asp Ile Ser Asn Thr Asp Asn 325 330 335			1124
45	TTT GTT AGA AAA AAC GAT CTA GCA TAC ATT AAT GTA GTA GAT CGT GGT Phe Val Arg Lys Asn Asp Leu Ala Tyr Ile Asn Val Val Asp Arg Gly 340 345 350 355			1172
50	GTA GAA TAT CGG TTA TAT GCT GAT ACA AAA TCA GAG AAA GAG AAA ATA Val Glu Tyr Arg Leu Tyr Ala Asp Thr Lys Ser Glu Lys Glu Lys Ile 360 365 370			1220
55	ATA AGA ACA TCT AAT CTA AAC GAT AGC TTA GGT CAA ATT ATA GTT ATG Ile Arg Thr Ser Asn Leu Asn Asp Ser Leu Gly Gln Ile Ile Val Met 375 380 385			1268
60	GAT TCA ATA GGA AAT AAT TGC ACA ATG AAT TTT CAA AAC AAT AAT GGG Asp Ser Ile Gly Asn Asn Cys Thr Met Asn Phe Gln Asn Asn Asn Gly 390 395 400			1316
65	AGC AAT ATA GGA TTA CTA GGT TTT CAT TCA AAT AAT TTG GTT GCT AGT Ser Asn Ile Gly Leu Leu Gly Phe His Ser Asn Asn Leu Val Ala Ser 405 410 415			1364
70	AGT TGG TAT TAT AAC AAT ATA CGA AGA AAT ACT AGC AGT AAT GGA TGC Ser Trp Tyr Tyr Asn Asn Ile Arg Arg Asn Thr Ser Ser Asn Gly Cys 420 425 430 435			1412
75	TTT TGG AGT TCT ATT TCT AAA GAG AAT GCA TGG AAA GAA TGAAAGCTT Phe Trp Ser Ser Ile Ser Lys Glu Asn Gly Trp Lys Glu 440 445			1460

## (2) INFORMATION FOR SEQ ID NO:73:

- 65 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 448 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear
- 70 (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:73:

5 Met Gly His His His His His His His His His His Ser Ser Gly His  
 1 5 10 15  
 Ile Glu Gly Arg His Met Ala Ser Met Ala Ile Leu Ile Ile Tyr Phe  
 20 25 30  
 10 Asn Arg Leu Tyr Lys Lys Ile Lys Asp Ser Ser Ile Leu Asp Met Arg  
 35 40 45  
 Tyr Glu Asn Asn Lys Phe Ile Asp Ile Ser Gly Tyr Gly Ser Asn Ile  
 50 55 60  
 15 Ser Ile Asn Gly Asn Val Tyr Ile Tyr Ser Thr Asn Arg Asn Gln Phe  
 65 70 75 80  
 Gly Ile Tyr Asn Ser Arg Leu Ser Glu Val Asn Ile Ala Gln Asn Asn  
 85 90 95  
 20 Asp Ile Ile Tyr Asn Ser Arg Tyr Gln Asn Phe Ser Ile Ser Phe Trp  
 100 105 110  
 25 Val Arg Ile Pro Lys His Tyr Lys Pro Met Asn His Asn Arg Glu Tyr  
 115 120 125  
 Thr Ile Ile Asn Cys Met Gly Asn Asn Asn Ser Gly Trp Lys Ile Ser  
 130 135 140  
 30 Leu Arg Thr Val Arg Asp Cys Glu Ile Ile Trp Thr Leu Gln Asp Thr  
 145 150 155 160  
 Ser Gly Asn Lys Glu Asn Leu Ile Phe Arg Tyr Glu Glu Leu Asn Arg  
 165 170 175  
 35 Ile Ser Asn Tyr Ile Asn Lys Trp Ile Phe Val Thr Ile Thr Asn Asn  
 180 185 190  
 40 Arg Leu Gly Asn Ser Arg Ile Tyr Ile Asn Gly Asn Leu Ile Val Glu  
 195 200 205  
 Lys Ser Ile Ser Asn Leu Gly Asp Ile His Val Ser Asp Asn Ile Leu  
 210 215 220  
 45 Phe Lys Ile Val Gly Cys Asp Asp Glu Thr Tyr Val Gly Ile Arg Tyr  
 225 230 235 240  
 Phe Lys Val Phe Asn Thr Glu Leu Asp Lys Thr Glu Ile Glu Thr Leu  
 245 250 255  
 50 Tyr Ser Asn Glu Pro Asp Pro Ser Ile Leu Lys Asn Tyr Trp Gly Asn  
 260 265 270  
 55 Tyr Leu Leu Tyr Asn Lys Lys Tyr Tyr Leu Phe Asn Leu Leu Arg Lys  
 275 280 285  
 Asp Lys Tyr Ile Thr Leu Asn Ser Gly Ile Leu Asn Ile Asn Gln Gln  
 290 295 300  
 60 Arg Gly Val Thr Glu Gly Ser Val Phe Leu Asn Tyr Lys Leu Tyr Glu  
 305 310 315 320  
 Gly Val Glu Val Ile Ile Arg Lys Asn Gly Pro Ile Asp Ile Ser Asn  
 325 330 335  
 65 Thr Asp Asn Phe Val Arg Lys Asn Asp Leu Ala Tyr Ile Asn Val Val  
 340 345 350  
 70 Asp Arg Gly Val Glu Tyr Arg Leu Tyr Ala Asp Thr Lys Ser Glu Lys  
 355 360 365

Glu Lys Ile Ile Arg Thr Ser Asn Leu Asn Asp Ser Leu Gly Gln Ile  
 370 375 380

5 Ile Val Met Asp Ser Ile Gly Asn Asn Cys Thr Met Asn Phe Gln Asn  
 385 390 395 400

Asn Asn Gly Ser Asn Ile Gly Leu Leu Gly Phe His Ser Asn Asn Leu  
 405 410 415

10 Val Ala Ser Ser Trp Tyr Tyr Asn Asn Ile Arg Arg Asn Thr Ser Ser  
 420 425 430

Asn Gly Cys Phe Trp Ser Ser Ile Ser Lys Glu Asn Gly Trp Lys Glu  
 435 440 445

15 (2) INFORMATION FOR SEQ ID NO:74:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 33 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: other nucleic acid  
 (A) DESCRIPTION: /desc = "DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:74:

30 CGCCATGGCT ATTCTAATTA TATATTTTAA TAG 33

(2) INFORMATION FOR SEQ ID NO:75:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 29 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: other nucleic acid  
 (A) DESCRIPTION: /desc = "DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:75:

45 GCAAGCTTTC ATTCTTTCCA TCCATTCTC 29

(2) INFORMATION FOR SEQ ID NO:76:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 3894 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear

50 (ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:  
 (A) NAME/KEY: CDS  
 (B) LOCATION: 1..3891

55 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:76:

60 ATG CCA GTT AAT ATA AAA AAC TTT AAT TAT AAT GAC CCT ATT AAT AAT 48  
 Met Pro Val Asn Ile Lys Asn Phe Asn Tyr Asn Asp Pro Ile Asn Asn  
 1 5 10 15

65 GAT GAC ATT ATT ATG ATG GAA CCA TTC AAT GAC CCA GGG CCA GGA ACA 96  
 Asp Asp Ile Ile Met Met Glu Pro Phe Asn Asp Pro Gly Pro Gly Thr  
 20 25 30

70 TAT TAT AAA GCT TTT AGG ATT ATA GAT CGT ATT TGG ATA GTA CCA GAA 144

	Tyr	Tyr	Lys	Ala	Phe	Arg	Ile	Ile	Asp	Arg	Ile	Trp	Ile	Val	Pro	Glu	
			35					40					45				
5	AGG	TTT	ACT	TAT	GGA	TTT	CAA	CCT	GAC	CAA	TTT	AAT	GCC	AGT	ACA	GGA	192
	Arg	Phe	Thr	Tyr	Gly	Phe	Gln	Pro	Asp	Gln	Phe	Asn	Ala	Ser	Thr	Gly	
		50					55					60					
10	GTT	TTT	AGT	AAA	GAT	GTC	TAC	GAA	TAT	TAC	GAT	CCA	ACT	TAT	TTA	AAA	240
	Val	Phe	Ser	Lys	Asp	Val	Tyr	Glu	Tyr	Tyr	Asp	Pro	Thr	Tyr	Leu	Lys	
		65				70					75				80		
15	ACC	GAT	GCT	GAA	AAA	GAT	AAA	TTT	TTA	AAA	ACA	ATG	ATT	AAA	TTA	TTT	288
	Thr	Asp	Ala	Glu	Lys	Asp	Lys	Phe	Leu	Lys	Thr	Met	Ile	Lys	Leu	Phe	
				85						90					95		
20	AAT	AGA	ATT	AAT	TCA	AAA	CCA	TCA	GGA	CAG	AGA	TTA	CTG	GAT	ATG	ATA	336
	Asn	Arg	Ile	Asn	Ser	Lys	Pro	Ser	Gly	Gln	Arg	Leu	Leu	Asp	Met	Ile	
				100					105					110			
25	GTA	GAT	GCT	ATA	CCT	TAT	CTT	GGA	AAT	GCA	TCT	ACA	CCG	CCC	GAC	AAA	384
	Val	Asp	Ala	Ile	Pro	Tyr	Leu	Gly	Asn	Ala	Ser	Thr	Pro	Pro	Asp	Lys	
			115					120					125				
30	TTT	GCA	GCA	AAT	GTT	GCA	AAT	GTA	TCT	ATT	AAT	AAA	AAA	ATT	ATC	CAA	432
	Phe	Ala	Ala	Asn	Val	Ala	Asn	Val	Ser	Ile	Asn	Lys	Lys	Ile	Ile	Gln	
			130				135					140					
35	CCT	GGA	GCT	GAA	GAT	CAA	ATA	AAA	GGT	TTA	ATG	ACA	AAT	TTA	ATA	ATA	480
	Pro	Gly	Ala	Glu	Asp	Gln	Ile	Lys	Gly	Leu	Met	Thr	Asn	Leu	Ile	Ile	
		145				150					155				160		
40	TTT	GGA	CCA	GGA	CCA	GTT	CTA	AGT	GAT	AAT	TTT	ACT	GAT	AGT	ATG	ATT	528
	Phe	Gly	Pro	Gly	Pro	Val	Leu	Ser	Asp	Asn	Phe	Thr	Asp	Ser	Met	Ile	
					165					170					175		
45	ATG	AAT	GGC	CAT	TCC	CCA	ATA	TCA	GAA	GGA	TTT	GGT	GCA	AGA	ATG	ATG	576
	Met	Asn	Gly	His	Ser	Pro	Ile	Ser	Glu	Gly	Phe	Gly	Ala	Arg	Met	Met	
				180					185					190			
50	ATA	AGA	TTT	TGT	CCT	AGT	TGT	TTA	AAT	GTA	TTT	AAT	AAT	GTT	CAG	GAA	624
	Ile	Arg	Phe	Cys	Pro	Ser	Cys	Leu	Asn	Val	Phe	Asn	Asn	Val	Gln	Glu	
				195				200					205				
55	AAT	AAA	GAT	ACA	TCT	ATA	TTT	AGT	AGA	CGC	GCG	TAT	TTT	GCA	GAT	CCA	672
	Asn	Lys	Asp	Thr	Ser	Ile	Phe	Ser	Arg	Arg	Ala	Tyr	Phe	Ala	Asp	Pro	
			210				215					220					
60	GCT	CTA	ACG	TTA	ATG	CAT	GAA	CTT	ATA	CAT	GTG	TTA	CAT	GGA	TTA	TAT	720
	Ala	Leu	Thr	Leu	Met	His	Glu	Leu	Ile	His	Val	Leu	His	Gly	Leu	Tyr	
		225				230					235				240		
65	GGA	ATT	AAG	ATA	AGT	AAT	TTA	CCA	ATT	ACT	CCA	AAT	ACA	AAA	GAA	TTT	768
	Gly	Ile	Lys	Ile	Ser	Asn	Leu	Pro	Ile	Thr	Pro	Asn	Thr	Lys	Glu	Phe	
					245					250					255		
70	TTC	ATG	CAA	CAT	AGC	GAT	CCT	GTA	CAA	GCA	GAA	GAA	CTA	TAT	ACA	TTC	816
	Phe	Met	Gln	His	Ser	Asp	Pro	Val	Gln	Ala	Glu	Glu	Leu	Tyr	Thr	Phe	
				260					265					270			
75	GGA	GGA	CAT	GAT	CCT	AGT	GTT	ATA	AGT	CCT	TCT	ACG	GAT	ATG	AAT	ATT	864
	Gly	Gly	His	Asp	Pro	Ser	Val	Ile	Ser	Pro	Ser	Thr	Asp	Met	Asn	Ile	
				275				280					285				
80	TAT	AAT	AAA	GCG	TTA	CAA	AAT	TTT	CAA	GAT	ATA	GCT	AAT	AGG	CTT	AAT	912
	Tyr	Asn	Lys	Ala	Leu	Gln	Asn	Phe	Gln	Asp	Ile	Ala	Asn	Arg	Leu	Asn	
			290				295					300					
85	ATT	GTT	TCA	AGT	GCC	CAA	GGG	AGT	GGA	ATT	GAT	ATT	TCC	TTA	TAT	AAA	960
	Ile	Val	Ser	Ser	Ala	Gln	Gly	Ser	Gly	Ile	Asp	Ile	Ser	Leu	Tyr	Lys	
						310					315					320	

	CAA ATA TAT AAA AAT AAA TAT GAT TTT GTT GAA GAT CCT AAT GGA AAA Gln Ile Tyr Lys Asn Lys Tyr Asp Phe Val Glu Asp Pro Asn Gly Lys	1008
	325 330 335	
5	TAT AGT GTA GAT AAG GAT AAG TTT GAT AAA TTA TAT AAG GCC TTA ATG Tyr Ser Val Asp Lys Asp Lys Phe Asp Lys Leu Tyr Lys Ala Leu Met	1056
	340 345 350	
10	TTT GGC TTT ACT GAA ACT AAT CTA GCT GGT GAA TAT GGA ATA AAA ACT Phe Gly Phe Thr Glu Thr Asn Leu Ala Gly Glu Tyr Gly Ile Lys Thr	1104
	355 360 365	
15	AGG TAT TCT TAT TTT AGT GAA TAT TTG CCA CCG ATA AAA ACT GAA AAA Arg Tyr Ser Tyr Phe Ser Glu Tyr Leu Pro Pro Ile Lys Thr Glu Lys	1152
	370 375 380	
20	TTG TTA GAC AAT ACA ATT TAT ACT CAA AAT GAA GGC TTT AAC ATA GCT Leu Leu Asp Asn Thr Ile Tyr Thr Gln Asn Glu Gly Phe Asn Ile Ala	1200
	385 390 395 400	
	AGT AAA AAT CTC AAA ACG GAA TTT AAT GGT CAG AAT AAG GCG GTA AAT Ser Lys Asn Leu Lys Thr Glu Phe Asn Gly Gln Asn Lys Ala Val Asn	1248
	405 410 415	
25	AAA GAG GCT TAT GAA GAA ATC AGC CTA GAA CAT CTC GTT ATA TAT AGA Lys Glu Ala Tyr Glu Glu Ile Ser Leu Glu His Leu Val Ile Tyr Arg	1296
	420 425 430	
30	ATA GCA ATG TGC AAG CCT GTA ATG TAC AAA AAT ACC GGT AAA TCT GAA Ile Ala Met Cys Lys Pro Val Met Tyr Lys Asn Thr Gly Lys Ser Glu	1344
	435 440 445	
35	CAG TGT ATT ATT GTT AAT AAT GAG GAT TTA TTT TTC ATA GCT AAT AAA Gln Cys Ile Ile Val Asn Asn Glu Asp Leu Phe Phe Ile Ala Asn Lys	1392
	450 455 460	
40	GAT AGT TTT TCA AAA GAT TTA GCT AAA GCA GAA ACT ATA GCA TAT AAT Asp Ser Phe Ser Lys Asp Leu Ala Lys Ala Glu Thr Ile Ala Tyr Asn	1440
	465 470 475 480	
	ACA CAA AAT AAT ACT ATA GAA AAT AAT TTT TCT ATA GAT CAG TTG ATT Thr Gln Asn Asn Thr Ile Glu Asn Asn Phe Ser Ile Asp Gln Leu Ile	1488
	485 490 495	
45	TTA GAT AAT GAT TTA AGC ACT GGC ATA GAC TTA CCA AAT GAA AAC ACA Leu Asp Asn Asp Leu Ser Ser Gly Ile Asp Leu Pro Asn Glu Asn Thr	1536
	500 505 510	
50	GAA CCA TTT ACA AAT TTT GAC GAC ATA GAT ATC CCT GTG TAT ATT AAA Glu Pro Phe Thr Asn Phe Asp Asp Ile Asp Ile Pro Val Tyr Ile Lys	1584
	515 520 525	
55	CAA TCT GCT TTA AAA AAA ATT TTT GTG GAT GGA GAT AGC CTT TTT GAA Gln Ser Ala Leu Lys Lys Ile Phe Val Asp Gly Asp Ser Leu Phe Glu	1632
	530 535 540	
60	TAT TTA CAT GCT CAA ACA TTT CCT TCT AAT ATA GAA AAT CTA CAA CTA Tyr Leu His Ala Gln Thr Phe Pro Ser Asn Ile Glu Asn Leu Gln Leu	1680
	545 550 555 560	
	ACG AAT TCA TTA AAT GAT GCT TTA AGA AAT AAT AAT AAA GTC TAT ACT Thr Asn Ser Leu Asn Asp Ala Leu Arg Asn Asn Asn Lys Val Tyr Thr	1728
	565 570 575	
65	TTT TTT TCT ACA AAC CTT GTT GAA AAA GCT AAT ACA GTT GTA GGT GCT Phe Phe Ser Thr Asn Leu Val Glu Lys Ala Asn Thr Val Val Gly Ala	1776
	580 585 590	
70	TCA CTT TTT GTA AAC TGG GTA AAA GGA GTA ATA GAT GAT TTT ACA TCT Ser Leu Phe Val Asn Trp Val Lys Gly Val Ile Asp Asp Phe Thr Ser	1824

	595	600	605	
5	GAA TCC ACA CAA AAA AGT ACT ATA GAT AAA GTT TCA GAT GTA TCC ATA Glu Ser Thr Gln Lys Ser Thr Ile Asp Lys Val Ser Asp Val Ser Ile 610 615 620			1872
10	ATT ATT CCC TAT ATA GGA CCT GCT TTG AAT GTA GGA AAT GAA ACA GCT Ile Ile Pro Tyr Ile Gly Pro Ala Leu Asn Val Gly Asn Glu Thr Ala 625 630 635 640			1920
15	AAA GAA AAT TTT AAA AAT GCT TTT GAA ATA GGT GGA GCC GCT ATC TTA Lys Glu Asn Phe Lys Asn Ala Phe Glu Ile Gly Gly Ala Ala Ile Leu 645 650 655			1968
20	ATG GAG TTT ATT CCA GAA CTT ATT GTA CCT ATA GTT GGA TTT TTT ACA Met Glu Phe Ile Pro Glu Leu Ile Val Pro Ile Val Gly Phe Phe Thr 660 665 670			2016
25	TTA GAA TCA TAT GTA GGA AAT AAA GGG CAT ATT ATT ATG ACG ATA TCC Leu Glu Ser Tyr Val Gly Asn Lys Gly His Ile Ile Met Thr Ile Ser 675 680 685			2064
30	AAT GCT TTA AAG AAA AGG GAT CAA AAA TGG ACA GAT ATG TAT GGT TTG Asn Ala Leu Lys Lys Arg Asp Gln Lys Trp Thr Asp Met Tyr Gly Leu 690 695 700			2112
35	ATA GTA TCG CAG TGG CTC TCA ACG GTT AAT ACT CAA TTT TAT ACA ATA Ile Val Ser Gln Trp Leu Ser Thr Val Asn Thr Gln Phe Tyr Thr Ile 705 710 715 720			2160
40	AAA GAA AGA ATG TAC AAT GCT TTA AAT AAT CAA TCA CAA GCA ATA GAA Lys Glu Arg Met Tyr Asn Ala Leu Asn Asn Gln Ser Gln Ala Ile Glu 725 730 735			2208
45	AAA ATA ATA GAA GAT CAA TAT AAT AGA TAT AGT GAA GAA GAT AAA ATG Lys Ile Ile Glu Asp Gln Tyr Asn Arg Tyr Ser Glu Glu Asp Lys Met 740 745 750			2256
50	AAT ATT AAC ATT GAT TTT AAT GAT ATA GAT TTT AAA CTT AAT CAA AGT Asn Ile Asn Ile Asp Phe Asn Asp Ile Asp Phe Lys Leu Asn Gln Ser 755 760 765			2304
55	ATA AAT TTA GCA ATA AAC AAT ATA GAT GAT TTT ATA AAC CAA TGT TCT Ile Asn Leu Ala Ile Asn Asn Ile Asp Asp Phe Ile Asn Gln Cys Ser 770 775 780			2352
60	ATA TCA TAT CTA ATG AAT AGA ATG ATT CCA TTA GCT GTA AAA AAG TTA Ile Ser Tyr Leu Met Asn Arg Met Ile Pro Leu Ala Val Lys Lys Leu 785 790 795 800			2400
65	AAA GAC TTT GAT GAT AAT CTT AAG AGA GAT TTA TTG GAG TAT ATA GAT Lys Asp Phe Asp Asp Asn Leu Lys Arg Asp Leu Leu Glu Tyr Ile Asp 805 810 815			2448
70	ACA AAT GAA CTA TAT TTA CTT GAT GAA GTA AAT ATT CTA AAA TCA AAA Thr Asn Glu Leu Tyr Leu Leu Asp Glu Val Asn Ile Leu Lys Ser Lys 820 825 830			2496
75	GTA AAT AGA CAC CTA AAA GAC AGT ATA CCA TTT GAT CTT TCA CTA TAT Val Asn Arg His Leu Lys Asp Ser Ile Pro Phe Asp Leu Ser Leu Tyr 835 840 845			2544
80	ACC AAG GAC ACA ATT TTA ATA CAA GTT TTT AAT AAT TAT ATT AGT AAT Thr Lys Asp Thr Ile Leu Ile Gln Val Phe Asn Asn Tyr Ile Ser Asn 850 855 860			2592
85	ATT AGT AGT AAT GCT ATT TTA AGT TTA AGT TAT AGA GGT GGG CGT TTA Ile Ser Ser Asn Ala Ile Leu Ser Leu Ser Tyr Arg Gly Gly Arg Leu 865 870 875 880			2640



	ATA GAT TCA TCT GGA TAT GGT GCA ACT ATG AAT GTA GGT TCA GAT GTT Ile Asp Ser Ser Gly Tyr Gly Ala Thr Met Asn Val Gly Ser Asp Val 885 890 895	2688
5	ATC TTT AAT GAT ATA GGA AAT GGT CAA TTT AAA TTA AAT AAT TCT GAA Ile Phe Asn Asp Ile Gly Asn Gly Gln Phe Lys Leu Asn Asn Ser Glu 900 905 910	2736
10	AAT AGT AAT ATT ACG GCA CAT CAA AGT AAA TTC GTT GTA TAT GAT AGT Asn Ser Asn Ile Thr Ala His Gln Ser Lys Phe Val Val Tyr Asp Ser 915 920 925	2784
15	ATG TTT GAT AAT TTT AGC ATT AAC TTT TGG GTA AGG ACT CCT AAA TAT Met Phe Asp Asn Phe Ser Ile Asn Phe Trp Val Arg Thr Pro Lys Tyr 930 935 940	2832
20	AAT AAT AAT GAT ATA CAA ACT TAT CTT CAA AAT GAG TAT ACA ATA ATT Asn Asn Asn Asp Ile Gln Thr Tyr Leu Gln Asn Glu Tyr Thr Ile Ile 945 950 955 960	2880
	AGT TGT ATA AAA AAT GAC TCA GGA TGG AAA GTA TCT ATT AAG GGA AAT Ser Cys Ile Lys Asn Asp Ser Gly Trp Lys Val Ser Ile Lys Gly Asn 965 970 975	2928
25	AGA ATA ATA TGG ACA TTA ATA GAT GTT AAT GCA AAA TCT AAA TCA ATA Arg Ile Ile Trp Thr Leu Ile Asp Val Asn Ala Lys Ser Lys Ser Ile 980 985 990	2976
30	TTT TTC CAA TAT AGT ATA AAA GAT AAT ATA TCA GAT TAT ATA AAT AAA Phe Phe Glu Tyr Ser Ile Lys Asp Asn Ile Ser Asp Tyr Ile Asn Lys 995 1000 1005	3024
35	TGG TTT TCC ATA ACT ATT ACT AAT GAT AGA TTA GGT AAC GCA AAT ATT Trp Phe Ser Ile Thr Ile Thr Asn Asp Arg Leu Gly Asn Ala Asn Ile 1010 1015 1020	3072
40	TAT ATA AAT GGA AGT TTG AAA AAA AGT GAA AAA ATT TTA AAC TTA GAT Tyr Ile Asn Gly Ser Leu Lys Lys Ser Glu Lys Ile Leu Asn Leu Asp 1025 1030 1035 1040	3120
	AGA ATT AAT TCT AGT AAT GAT ATA GAC TTC AAA TTA ATT AAT TGT ACA Arg Ile Asn Ser Ser Asn Asp Ile Asp Phe Lys Leu Ile Asn Cys Thr 1045 1050 1055	3168
45	GAT ACT ACT AAA TTT GTT TGG ATT AAG GAT TTT AAT ATT TTT GGT AGA Asp Thr Thr Lys Phe Val Trp Ile Lys Asp Phe Asn Ile Phe Gly Arg 1060 1065 1070	3216
50	GAA TTA AAT GCT ACA GAA GTA TCT TCA CTA TAT TGG ATT CAA TCA TCT Glu Leu Asn Ala Thr Glu Val Ser Ser Leu Tyr Trp Ile Gln Ser Ser 1075 1080 1085	3264
55	ACA AAT ACT TTA AAA GAT TTT TGG GGG AAT CCT TTA AGA TAC GAT ACA Thr Asn Thr Leu Lys Asp Phe Trp Gly Asn Pro Leu Arg Tyr Asp Thr 1090 1095 1100	3312
60	CAA TAC TAT CTG TTT AAT CAA GGT ATG CAA AAT ATC TAT ATA AAG TAT Gln Tyr Tyr Leu Phe Asn Gln Gly Met Gln Asn Ile Tyr Ile Lys Tyr 1105 1110 1115 1120	3360
	TTT AGT AAA GCT TCT ATG GGG GAA ACT GCA CCA CGT ACA AAC TTT AAT Phe Ser Lys Ala Ser Met Gly Glu Thr Ala Pro Arg Thr Asn Phe Asn 1125 1130 1135	3408

	AAT GCA GCA ATA AAT TAT CAA AAT TTA TAT CTT GGT TTA CGA TTT ATT Asn Ala Ala Ile Asn Tyr Gln Asn Leu Tyr Leu Gly Leu Arg Phe Ile 1140 1145 1150	3456
5	ATA AAA AAA GCA TCA AAT TCT CGG AAT ATA AAT AAT GAT AAT ATA GTC Ile Lys Lys Ala Ser Asn Ser Arg Asn Ile Asn Asn Asp Asn Ile Val 1155 1160 1165	3504
10	AGA GAA GGA GAT TAT ATA TAT CTT AAT ATT GAT AAT ATT TCT GAT GAA Arg Glu Gly Asp Tyr Ile Tyr Leu Asn Ile Asp Asn Ile Ser Asp Glu 1170 1175 1180	3552
15	TCT TAC AGA GTA TAT GTT TTG GTG AAT TCT AAA GAA ATT CAA ACT CAA Ser Tyr Arg Val Tyr Val Leu Val Asn Ser Lys Glu Ile Gln Thr Gln 1185 1190 1195 1200	3600
20	TTA TTT TTA GCA CCC ATA AAT GAT GAT CCT ACG TTC TAT GAT GTA CTA Leu Phe Leu Ala Pro Ile Asn Asp Asp Pro Thr Phe Tyr Asp Val Leu 1205 1210 1215	3648
	CAA ATA AAA AAA TAT TAT GAA AAA ACA ACA TAT AAT TGT CAG ATA CTT Gln Ile Lys Lys Tyr Tyr Glu Lys Thr Thr Tyr Asn Cys Gln Ile Leu 1220 1225 1230	3696
25	TGC GAA AAA GAT ACT AAA ACA TTT GGG CTG TTT GGA ATT GGT AAA TTT Cys Glu Lys Asp Thr Lys Thr Phe Gly Leu Phe Gly Ile Gly Lys Phe 1235 1240 1245	3744
30	GTT AAA GAT TAT GGA TAT GTT TGG GAT ACC TAT GAT AAT TAT TTT TGC Val Lys Asp Tyr Gly Tyr Val Trp Asp Thr Tyr Asp Asn Tyr Phe Cys 1250 1255 1260	3792
35	ATA AGT CAG TGG TAT CTC AGA AGA ATA TCT GAA AAT ATA AAT AAA TTA Ile Ser Gln Trp Tyr Leu Arg Arg Ile Ser Glu Asn Ile Asn Lys Leu 1265 1270 1275 1280	3840
40	AGG TTG GGA TGT AAT TGG CAA TTC ATT CCC GTG GAT GAA GGA TGG ACA Arg Leu Gly Cys Asn Trp Gln Phe Ile Pro Val Asp Glu Gly Trp Thr 1285 1290 1295	3888
	GAA TAA Glu	3894
45	(2) INFORMATION FOR SEQ ID NO:77:	
	(i) SEQUENCE CHARACTERISTICS:	
	(A) LENGTH: 1297 amino acids	
	(B) TYPE: amino acid	
	(D) TOPOLOGY: linear	
50	(ii) MOLECULE TYPE: protein	
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:77:	
55	Met Pro Val Asn Ile Lys Asn Phe Asn Tyr Asn Asp Pro Ile Asn Asn 1 5 10 15	
60	Asp Asp Ile Ile Met Met Glu Pro Phe Asn Asp Pro Gly Pro Gly Thr 20 25 30	
	Tyr Tyr Lys Ala Phe Arg Ile Ile Asp Arg Ile Trp Ile Val Pro Glu 35 40 45	
65	Arg Phe Thr Tyr Gly Phe Gln Pro Asp Gln Phe Asn Ala Ser Thr Gly 50 55 60	
	Val Phe Ser Lys Asp Val Tyr Glu Tyr Tyr Asp Pro Thr Tyr Leu Lys 65 70 75 80	
70	Thr Asp Ala Glu Lys Asp Lys Phe Leu Lys Thr Met Ile Lys Leu Phe	

	85	90	95
5	Asn Arg Ile Asn Ser Lys Pro Ser Gly Gln Arg Leu Leu Asp Met Ile 100 105 110		
	Val Asp Ala Ile Pro Tyr Leu Gly Asn Ala Ser Thr Pro Pro Asp Lys 115 120 125		
10	Phe Ala Ala Asn Val Ala Asn Val Ser Ile Asn Lys Lys Ile Ile Gln 130 135 140		
	Pro Gly Ala Glu Asp Gln Ile Lys Gly Leu Met Thr Asn Leu Ile Ile 145 150 155 160		
15	Phe Gly Pro Gly Pro Val Leu Ser Asp Asn Phe Thr Asp Ser Met Ile 165 170 175		
	Met Asn Gly His Ser Pro Ile Ser Glu Gly Phe Gly Ala Arg Met Met 180 185 190		
20	Ile Arg Phe Cys Pro Ser Cys Leu Asn Val Phe Asn Asn Val Gln Glu 195 200 205		
	Asn Lys Asp Thr Ser Ile Phe Ser Arg Arg Ala Tyr Phe Ala Asp Pro 210 215 220		
	Ala Leu Thr Leu Met His Glu Leu Ile His Val Leu His Gly Leu Tyr 225 230 235 240		
30	Gly Ile Lys Ile Ser Asn Leu Pro Ile Thr Pro Asn Thr Lys Glu Phe 245 250 255		
	Phe Met Gln His Ser Asp Pro Val Gln Ala Glu Glu Leu Tyr Thr Phe 260 265 270		
35	Gly Gly His Asp Pro Ser Val Ile Ser Pro Ser Thr Asp Met Asn Ile 275 280 285		
	Tyr Asn Lys Ala Leu Gln Asn Phe Gln Asp Ile Ala Asn Arg Leu Asn 290 295 300		
40	Ile Val Ser Ser Ala Gln Gly Ser Gly Ile Asp Ile Ser Leu Tyr Lys 305 310 315 320		
45	Gln Ile Tyr Lys Asn Lys Tyr Asp Phe Val Glu Asp Pro Asn Gly Lys 325 330 335		
	Tyr Ser Val Asp Lys Asp Lys Phe Asp Lys Leu Tyr Lys Ala Leu Met 340 345 350		
50	Phe Gly Phe Thr Glu Thr Asn Leu Ala Gly Glu Tyr Gly Ile Lys Thr 355 360 365		
	Arg Tyr Ser Tyr Phe Ser Glu Tyr Leu Pro Pro Ile Lys Thr Glu Lys 370 375 380		
	Leu Leu Asp Asn Thr Ile Tyr Thr Gln Asn Glu Gly Phe Asn Ile Ala 385 390 395 400		
60	Ser Lys Asn Leu Lys Thr Glu Phe Asn Gly Gln Asn Lys Ala Val Asn 405 410 415		
	Lys Glu Ala Tyr Glu Glu Ile Ser Leu Glu His Leu Val Ile Tyr Arg 420 425 430		
65	Ile Ala Met Cys Lys Pro Val Met Tyr Lys Asn Thr Gly Lys Ser Glu 435 440 445		
70	Gln Cys Ile Ile Val Asn Asn Glu Asp Leu Phe Phe Ile Ala Asn Lys 450 455 460		

Asp Ser Phe Ser Lys Asp Leu Ala Lys Ala Glu Thr Ile Ala Tyr Asn  
 465 470 475 480  
 5 Thr Gln Asn Asn Thr Ile Glu Asn Asn Phe Ser Ile Asp Gln Leu Ile  
 485 490 495  
 Leu Asp Asn Asp Leu Ser Ser Gly Ile Asp Leu Pro Asn Glu Asn Thr  
 500 505 510  
 10 Glu Pro Phe Thr Asn Phe Asp Asp Ile Asp Ile Pro Val Tyr Ile Lys  
 515 520 525  
 Gln Ser Ala Leu Lys Lys Ile Phe Val Asp Gly Asp Ser Leu Phe Glu  
 530 535 540  
 15 Tyr Leu His Ala Gln Thr Phe Pro Ser Asn Ile Glu Asn Leu Gln Leu  
 545 550 555 560  
 20 Thr Asn Ser Leu Asn Asp Ala Leu Arg Asn Asn Asn Lys Val Tyr Thr  
 565 570 575  
 Phe Phe Ser Thr Asn Leu Val Glu Lys Ala Asn Thr Val Val Gly Ala  
 580 585 590  
 25 Ser Leu Phe Val Asn Trp Val Lys Gly Val Ile Asp Asp Phe Thr Ser  
 595 600 605  
 Glu Ser Thr Gln Lys Ser Thr Ile Asp Lys Val Ser Asp Val Ser Ile  
 610 615 620  
 30 Ile Ile Pro Tyr Ile Gly Pro Ala Leu Asn Val Gly Asn Glu Thr Ala  
 625 630 635 640  
 35 Lys Glu Asn Phe Lys Asn Ala Phe Glu Ile Gly Gly Ala Ala Ile Leu  
 645 650 655  
 Met Glu Phe Ile Pro Glu Leu Ile Val Pro Ile Val Gly Phe Phe Thr  
 660 665 670  
 40 Leu Glu Ser Tyr Val Gly Asn Lys Gly His Ile Ile Met Thr Ile Ser  
 675 680 685  
 Asn Ala Leu Lys Lys Arg Asp Gln Lys Trp Thr Asp Met Tyr Gly Leu  
 690 695 700  
 45 Ile Val Ser Gln Trp Leu Ser Thr Val Asn Thr Gln Phe Tyr Thr Ile  
 705 710 715 720  
 50 Lys Glu Arg Met Tyr Asn Ala Leu Asn Asn Gln Ser Gln Ala Ile Glu  
 725 730 735  
 Lys Ile Ile Glu Asp Gln Tyr Asn Arg Tyr Ser Glu Glu Asp Lys Met  
 740 745 750  
 55 Asn Ile Asn Ile Asp Phe Asn Asp Ile Asp Phe Lys Leu Asn Gln Ser  
 755 760 765  
 Ile Asn Leu Ala Ile Asn Asn Ile Asp Asp Phe Ile Asn Gln Cys Ser  
 770 775 780  
 60 Ile Ser Tyr Leu Met Asn Arg Met Ile Pro Leu Ala Val Lys Lys Leu  
 785 790 795 800  
 65 Lys Asp Phe Asp Asp Asn Leu Lys Arg Asp Leu Leu Glu Tyr Ile Asp  
 805 810 815  
 Thr Asn Glu Leu Tyr Leu Leu Asp Glu Val Asn Ile Leu Lys Ser Lys  
 820 825 830  
 70 Val Asn Arg His Leu Lys Asp Ser Ile Pro Phe Asp Leu Ser Leu Tyr

	835	840	845
5	Thr Lys Asp Thr Ile Leu 850	Ile Gln Val Phe Asn 855	Asn Tyr Ile Ser Asn 860
	Ile Ser Ser Asn Ala Ile 865	Leu Ser Leu Ser Tyr 870	Arg Gly Gly Arg Leu 875 880
10	Ile Asp Ser Ser Gly Tyr 885	Gly Ala Thr Met 890	Asn Val Gly Ser Asp Val 895
	Ile Phe Asn Asp Ile 900	Gly Asn Gly Gln Phe 905	Lys Leu Asn Asn Ser Glu 910
15	Asn Ser Asn Ile Thr Ala 915	His Gln Ser Lys Phe 920	Val Val Tyr Asp Ser 925
20	Met Phe Asp Asn Phe Ser 930	Ile Asn Phe Trp Val 935	Arg Thr Pro Lys Tyr 940
	Asn Asn Asn Asp Ile 945	Gln Thr Tyr Leu Gln 950	Asn Glu Tyr Thr Ile Ile 955 960
25	Ser Cys Ile Lys Asn Asp 965	Ser Gly Trp Lys Val 970	Ser Ile Lys Gly Asn 975
	Arg Ile Ile Trp Thr 980	Leu Ile Asp Val Asn 985	Ala Lys Ser Lys Ser Ile 990
30	Phe Phe Glu Tyr Ser Ile 995	Lys Asp Asn Ile Ser 1000	Asp Tyr Ile Asn Lys 1005
35	Trp Phe Ser Ile Thr Ile 1010	Thr Asn Asp Arg Leu 1015	Gly Asn Ala Asn Ile 1020
	Tyr Ile Asn Gly Ser Leu 1025	Lys Lys Ser Glu Lys 1030	Ile Leu Asn Leu Asp 1035 1040
40	Arg Ile Asn Ser Ser Asn 1045	Asp Ile Asp Phe Lys 1050	Leu Ile Asn Cys Thr 1055
	Asp Thr Thr Lys Phe Val 1060	Trp Ile Lys Asp Phe 1065	Asn Ile Phe Gly Arg 1070
45	Glu Leu Asn Ala Thr Glu 1075	Val Ser Ser Leu Tyr 1080	Trp Ile Gln Ser Ser 1085
50	Thr Asn Thr Leu Lys Asp 1090	Phe Trp Gly Asn Pro 1095	Leu Arg Tyr Asp Thr 1100
	Gln Tyr Tyr Leu Phe Asn 1105	Gln Gly Met Gln Asn 1110	Ile Tyr Ile Lys Tyr 1115 1120
55	Phe Ser Lys Ala Ser Met 1125	Gly Glu Thr Ala Pro 1130	Arg Thr Asn Phe Asn 1135
	Asn Ala Ala Ile Asn Tyr 1140	Gln Asn Leu Tyr Leu 1145	Gly Leu Arg Phe Ile 1150
60	Ile Lys Lys Ala Ser Asn 1155	Ser Arg Asn Ile Asn 1160	Asn Asp Asn Ile Val 1165
65	Arg Glu Gly Asp Tyr Ile 1170	Tyr Leu Asn Ile Asp 1175	Asn Ile Ser Asp Glu 1180
	Ser Tyr Arg Val Tyr Val 1185	Leu Val Asn Ser Lys 1190	Glu Ile Gln Thr Gln 1195 1200
70	Leu Phe Leu Ala Pro Ile 1205	Asn Asp Asp Pro Thr 1210	Phe Tyr Asp Val Leu 1215

	Gln Ile Lys Lys Tyr Tyr Glu Lys Thr Thr Tyr Asn Cys Gln Ile Leu	
	1220 1225 1230	
5	Cys Glu Lys Asp Thr Lys Thr Phe Gly Leu Phe Gly Ile Gly Lys Phe	
	1235 1240 1245	
	Val Lys Asp Tyr Gly Tyr Val Trp Asp Thr Tyr Asp Asn Tyr Phe Cys	
	1250 1255 1260	
10	Ile Ser Gln Trp Tyr Leu Arg Arg Ile Ser Glu Asn Ile Asn Lys Leu	
	1265 1270 1275 1280	
	Arg Leu Gly Cys Asn Trp Gln Phe Ile Pro Val Asp Glu Gly Trp Thr	
15	1285 1290 1295	
	Glu	
	(2) INFORMATION FOR SEQ ID NO:78:	
20	(i) SEQUENCE CHARACTERISTICS:	
	(A) LENGTH: 1535 base pairs	
	(B) TYPE: nucleic acid	
	(C) STRANDEDNESS: double	
25	(D) TOPOLOGY: linear	
	(ii) MOLECULE TYPE: DNA (genomic)	
	(ix) FEATURE:	
30	(A) NAME/KEY: CDS	
	(B) LOCATION: 108..1526	
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:78:	
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	Met Gly His	
	1	
40	CAT CAT CAT CAT CAT CAT CAT CAC AGC AGC GGC CAT ATC GAA GGT	164
	His His His His His His His His His Ser Ser Gly His Ile Glu Gly	
	5 10 15	
45	CGT CAT ATG GCT AGC ATG GCT GAC ACA ATT TTA ATA CAA GTT TTT AAT	212
	Arg His Met Ala Ser Met Ala Asp Thr Ile Leu Ile Gln Val Phe Asn	
	20 25 30 35	
	AAT TAT ATT AGT AAT ATT AGT AGT AAT GCT ATT TTA AGT TTA AGT TAT	260
50	Asn Tyr Ile Ser Asn Ile Ser Ser Asn Ala Ile Leu Ser Leu Ser Tyr	
	40 45 50	
	AGA GGT GGG CGT TTA ATA GAT TCA TCT GGA TAT GGT GCA ACT ATG AAT	308
55	Arg Gly Gly Arg Leu Ile Asp Ser Ser Gly Tyr Gly Ala Thr Met Asn	
	55 60 65	
	GTA GGT TCA GAT GTT ATC TTT AAT GAT ATA GGA AAT GGT CAA TTT AAA	356
	Val Gly Ser Asp Val Ile Phe Asn Asp Ile Gly Asn Gly Gln Phe Lys	
	70 75 80	
60	TTA AAT AAT TCT GAA AAT AGT AAT ATT ACG GCA CAT CAA AGT AAA TTC	404
	Leu Asn Asn Ser Glu Asn Ser Asn Ile Thr Ala His Gln Ser Lys Phe	
	85 90 95	
65	GTT GTA TAT GAT AGT ATG TTT GAT AAT TTT AGC ATT AAC TTT TGG GTA	452
	Val Val Tyr Asp Ser Met Phe Asp Asn Phe Ser Ile Asn Phe Trp Val	
	100 105 110 115	
70	AGG ACT CCT AAA TAT AAT AAT AAT GAT ATA CAA ACT TAT CTT CAA AAT	500
	Arg Thr Pro Lys Tyr Asn Asn Asn Asp Ile Gln Thr Tyr Leu Gln Asn	
	120 125 130	

	GAG TAT ACA ATA ATT AGT TGT ATA AAA AAT GAC TCA GGA TGG AAA GTA Glu Tyr Thr Ile Ile Ser Cys Ile Lys Asn Asp Ser Gly Trp Lys Val	548
	135 140 145	
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10	AAA TCT AAA TCA ATA TTT TTC GAA TAT AGT ATA AAA GAT AAT ATA TCA Lys Ser Lys Ser Ile Phe Phe Glu Tyr Ser Ile Lys Asp Asn Ile Ser	644
	165 170 175	
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20 (2) INFORMATION FOR SEQ ID NO:79:

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 (A) LENGTH: 473 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

25 (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:79:

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Ile Glu Gly Arg His Met Ala Ser Met Ala Asp Thr Ile Leu Ile Gln  
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 35 40 45

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Thr Met Asn Val Gly Ser Asp Val Ile Phe Asn Asp Ile Gly Asn Gly  
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45 Gln Phe Lys Leu Asn Asn Ser Glu Asn Ser Asn Ile Thr Ala His Gln  
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Ser Lys Phe Val Val Tyr Asp Ser Met Phe Asp Asn Phe Ser Ile Asn  
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 5 Lys Asp Phe Asn Ile Phe Gly Arg Glu Leu Asn Ala Thr Glu Val Ser  
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 275 280 285  
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 290 295 300  
 15 Thr Ala Pro Arg Thr Asn Phe Asn Asn Ala Ala Ile Asn Tyr Gln Asn  
 305 310 315 320  
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 325 330 335  
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 405 410 415  
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 420 425 430  
 40 Asp Thr Tyr Asp Asn Tyr Phe Cys Ile Ser Gln Trp Tyr Leu Arg Arg  
 435 440 445  
 Ile Ser Glu Asn Ile Asn Lys Leu Arg Leu Gly Cys Asn Trp Gln Phe  
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 465 470

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 55 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear  
 (ii) MOLECULE TYPE: other nucleic acid  
 (A) DESCRIPTION: /desc = "DNA"  
 60 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:80:

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30

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 70 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "DNA"

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(2) INFORMATION FOR SEQ ID NO:82:

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(A) LENGTH: 12 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: not relevant  
15 (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:  
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(B) LOCATION: 12  
(D) OTHER INFORMATION: /note= "The asparagine residue at  
this position contains an amide group."

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:82:  
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## CLAIMS

1. A host cell containing a recombinant expression vector, said vector encoding a protein comprising at least a portion of a *Clostridium botulinum* toxin, said toxin selected from the group consisting of type B toxin and type E toxin.
2. The host cell of Claim 1, wherein said host cell is capable of expressing said protein at a level greater than or equal to 5% of the total cellular protein.
3. The host cell of Claim 1, wherein said host cell is capable of expressing said protein as a soluble protein at a level greater than or equal to 0.25% of the total soluble cellular protein.
4. The host cell of Claim 1, wherein said host cell is an *Escherichia coli* cell.
5. The host cell of Claim 1, wherein said host cell is an insect cell.
6. The host cell of Claim 1, wherein said host cell is a yeast cell.
7. A host cell containing a recombinant expression vector, said vector encoding a fusion protein comprising a non-toxin protein sequence and at least a portion of a *Clostridium botulinum* toxin, said toxin selected from the group consisting of type B toxin and type E toxin.
8. The host cell of Claim 7, wherein said portion of said toxin comprises the receptor binding domain.
9. The host cell of Claim 7, wherein said non-toxin protein sequence comprises a poly-histidine tract.
10. A vaccine comprising a fusion protein, said fusion protein comprising a non-toxin protein sequence and at least a portion of a *Clostridium botulinum* toxin, said toxin selected from the group consisting of type B toxin and type E toxin.

11. The vaccine of Claim 10 further comprising a fusion protein comprising a non-toxin protein sequence and at least a portion of *Clostridium botulinum* type A toxin.

12. The vaccine of Claim 10, wherein said portion of said *Clostridium botulinum* toxin comprises the receptor binding domain.

13. The vaccine of Claim 10 wherein said non-toxin protein sequence comprises a poly-histidine tract.

14. The vaccine of Claim 10, wherein said vaccine is substantially endotoxin-free.

15. A method of generating antibody directed against a *Clostridium botulinum* toxin comprising:

a) providing in any order:

i) an antigen comprising a fusion protein comprising a non-toxin protein sequence and at least a portion of a *Clostridium botulinum* toxin, said toxin selected from the group consisting of type B toxin and type E toxin, and

ii) a host; and

b) immunizing said host with said antigen so as to generate an antibody.

16. The method of Claim 15, wherein said antigen further comprises a fusion protein comprising a non-toxin protein sequence and at least a portion of *Clostridium botulinum* type A toxin.

17. The method of Claim 15, wherein said portion of said *Clostridium botulinum* toxin comprises the receptor binding domain.

18. The method of Claim 15 wherein said non-toxin protein sequence comprises a poly-histidine tract.

19. The method of Claim 15 wherein said host is a mammal.

20. The method of Claim 19 wherein said mammal is a human.

21. The method of Claim 15 further comprising step c) collecting said antibodies from said host.

22. The method of Claim 21 further comprising step d) purifying said antibodies.

23. The antibody raised according to the method of Claim 15.

24. The antibody raised according to the method of Claim 16.

FIGURE 1

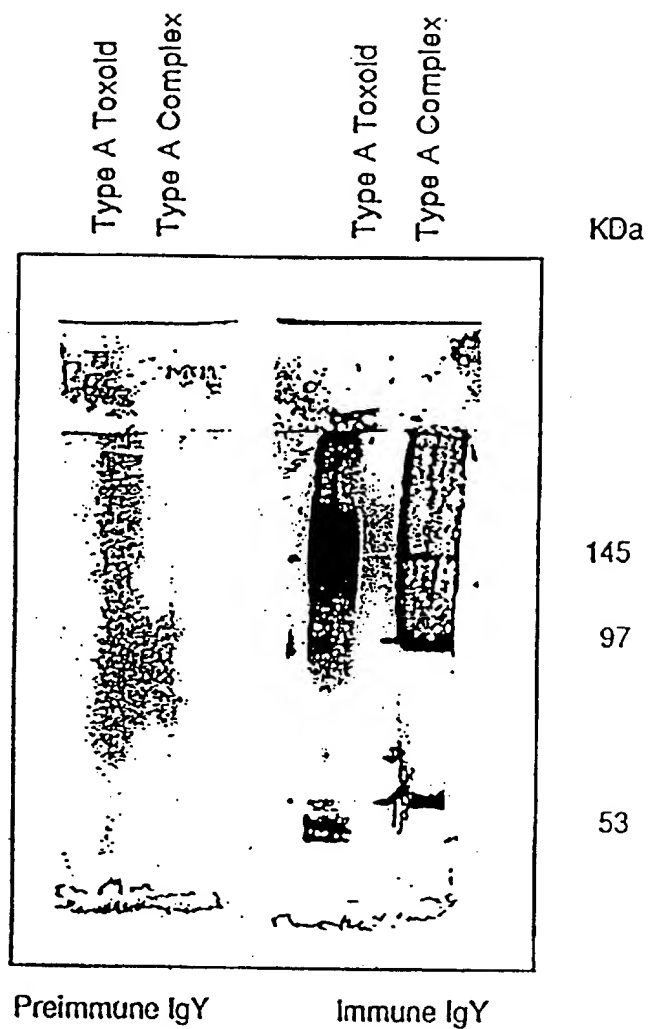


FIGURE 2

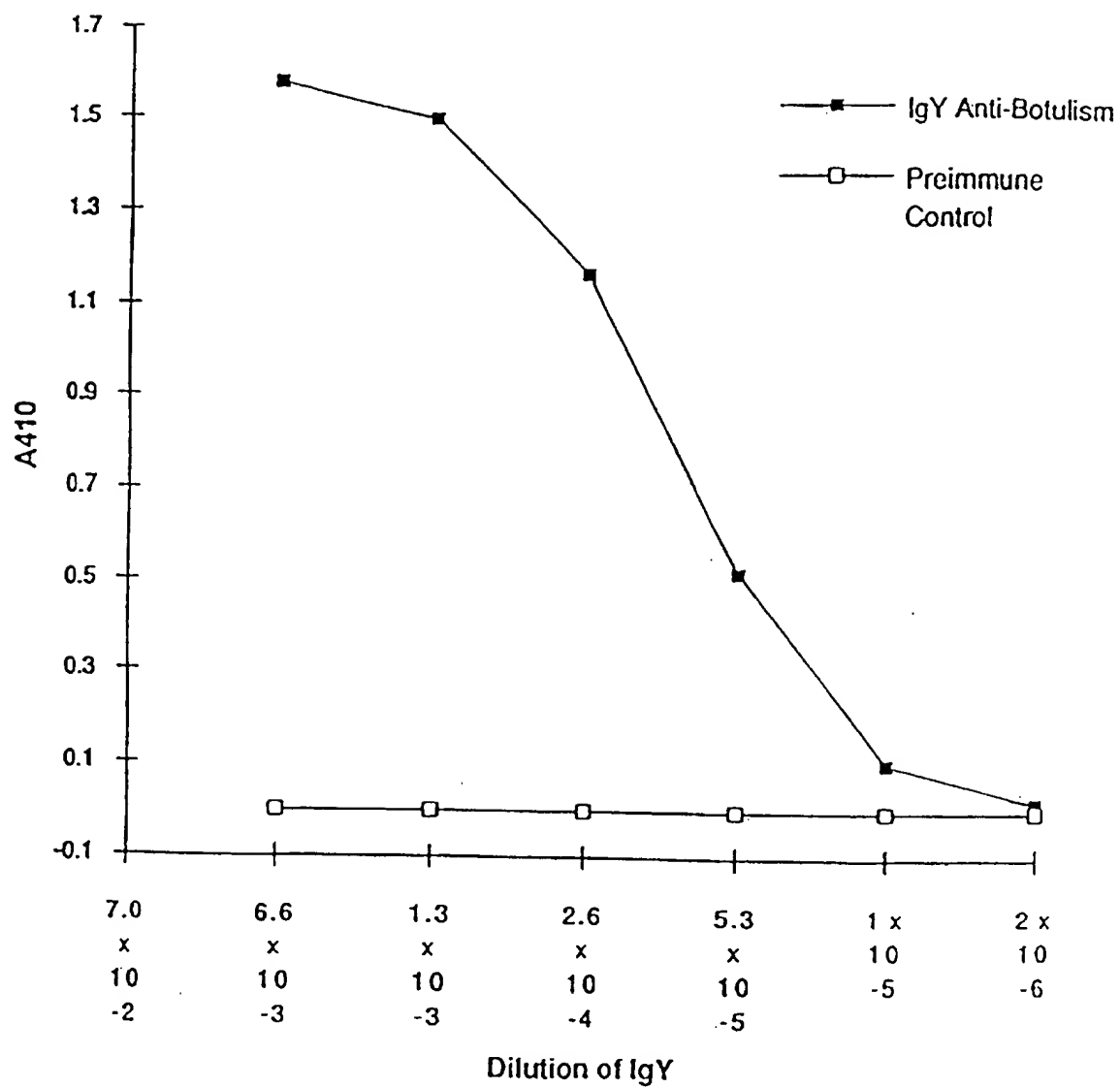


FIGURE 3

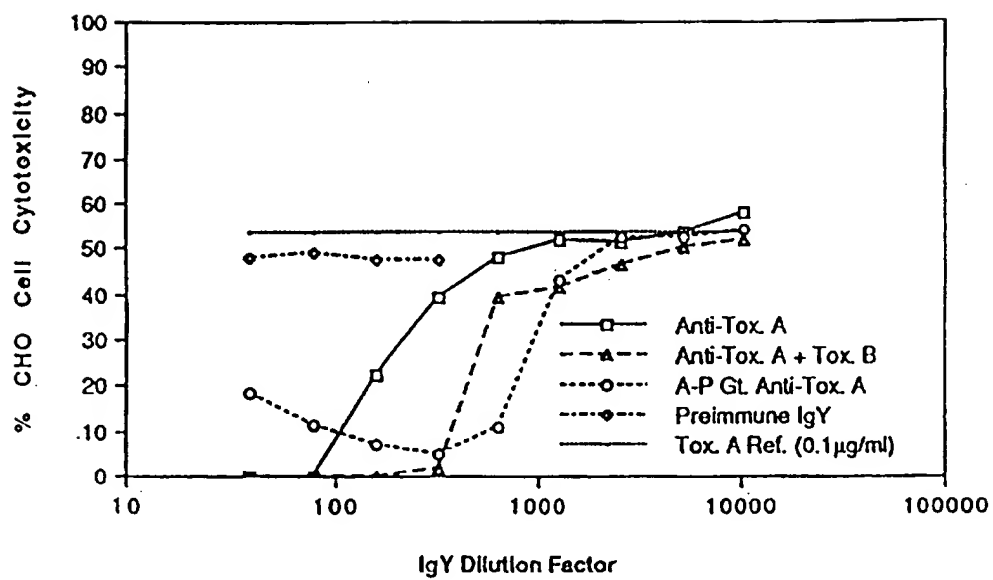




FIGURE 4

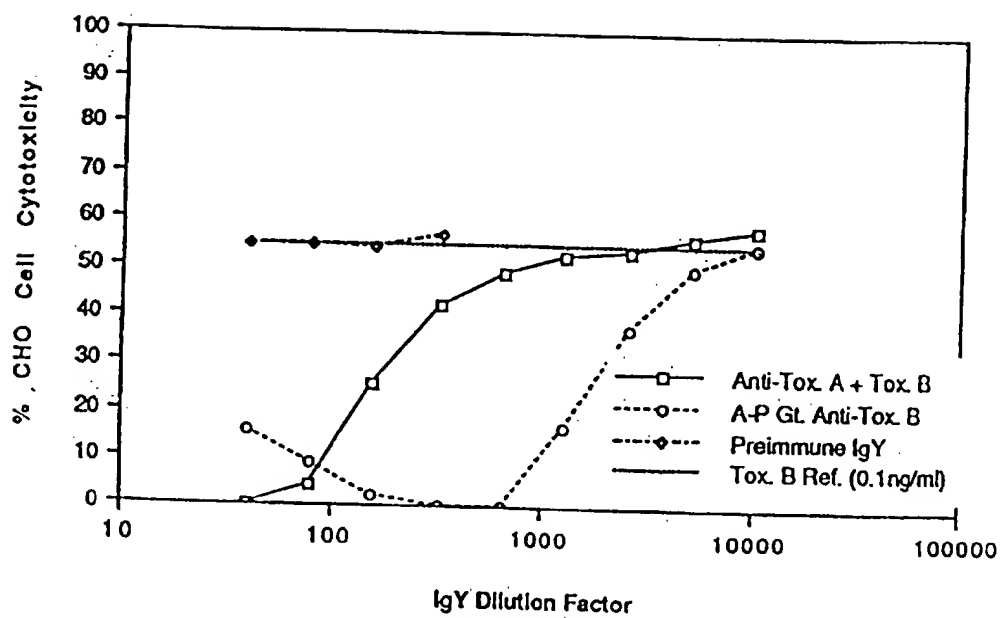


FIGURE 5

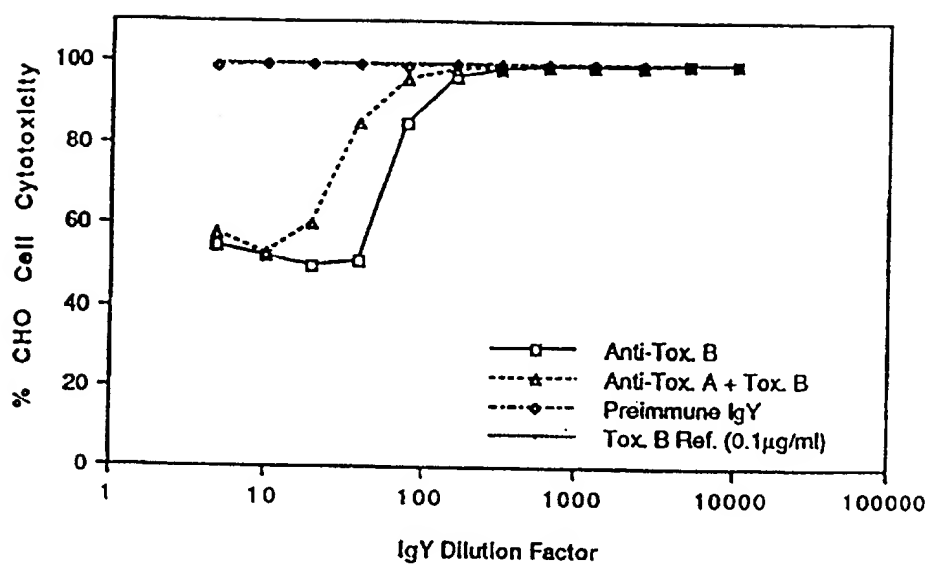


FIGURE 6

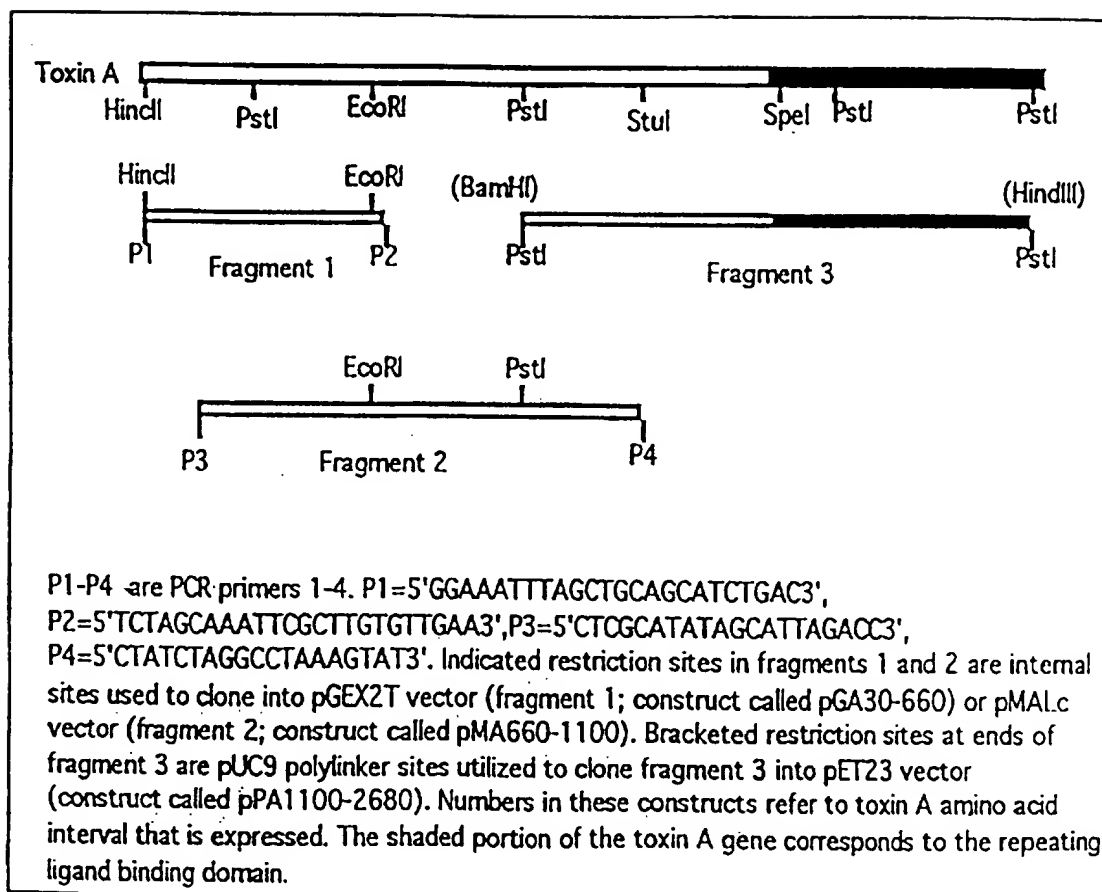


FIGURE 7

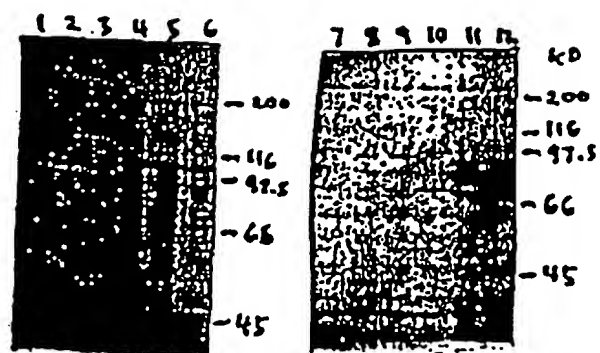


FIGURE 8

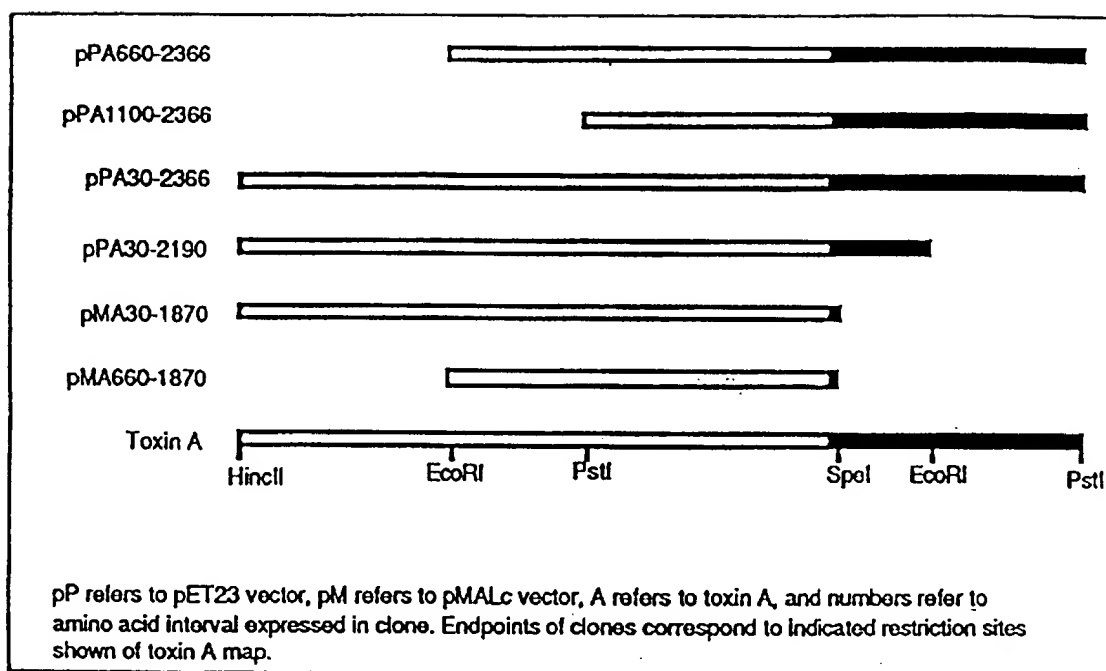


FIGURE 9

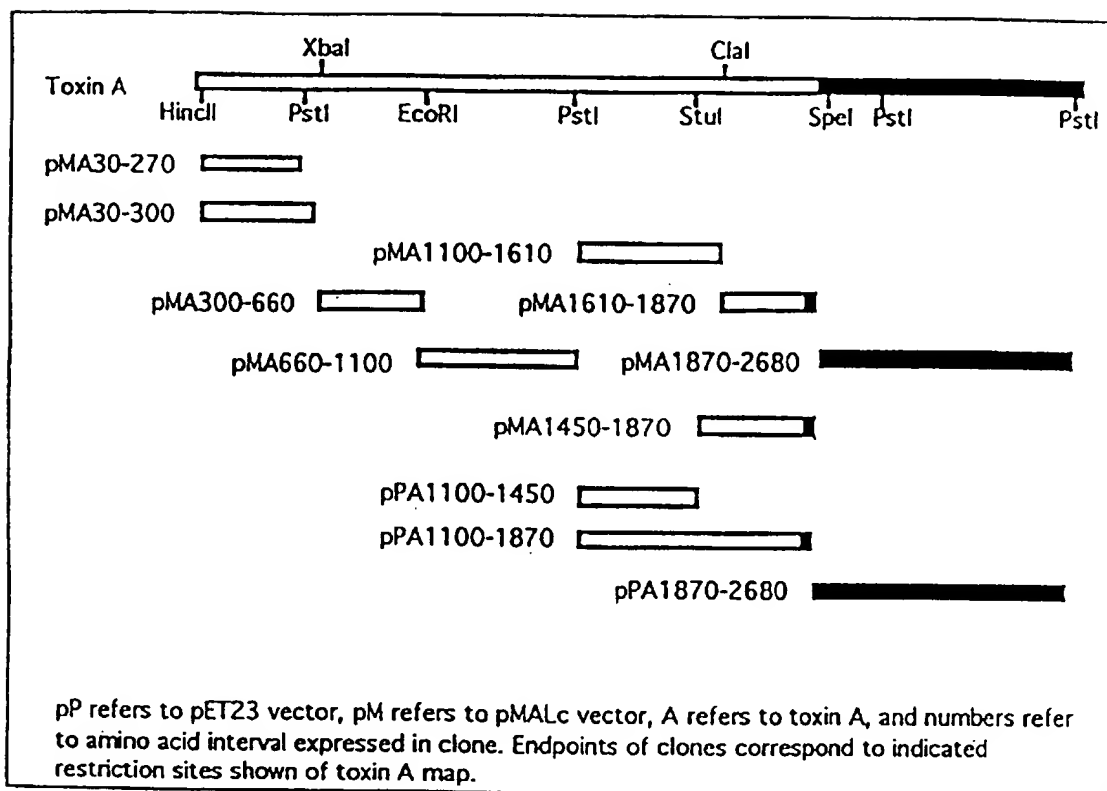


FIGURE 10

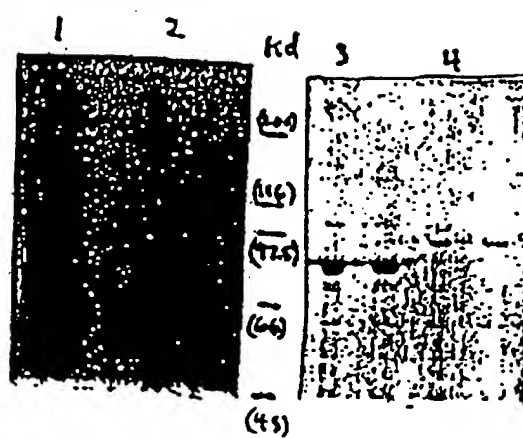


FIGURE 11

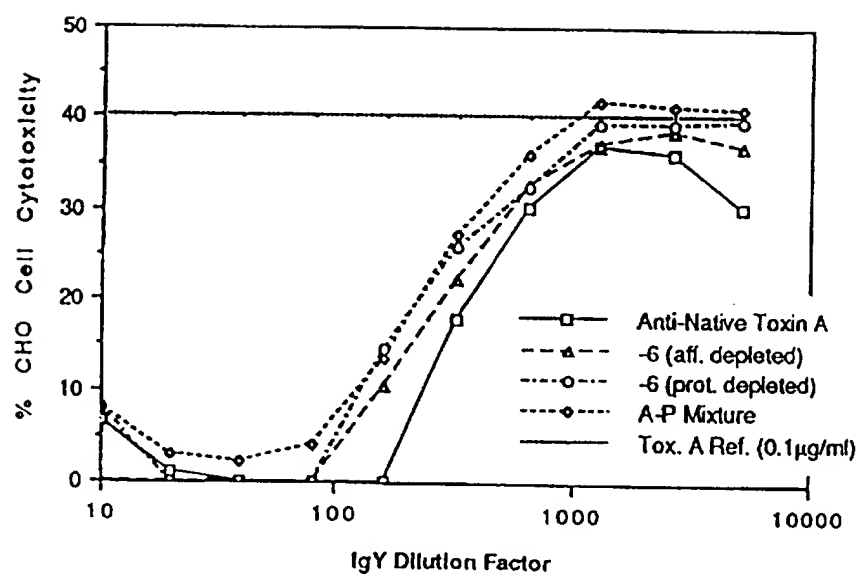
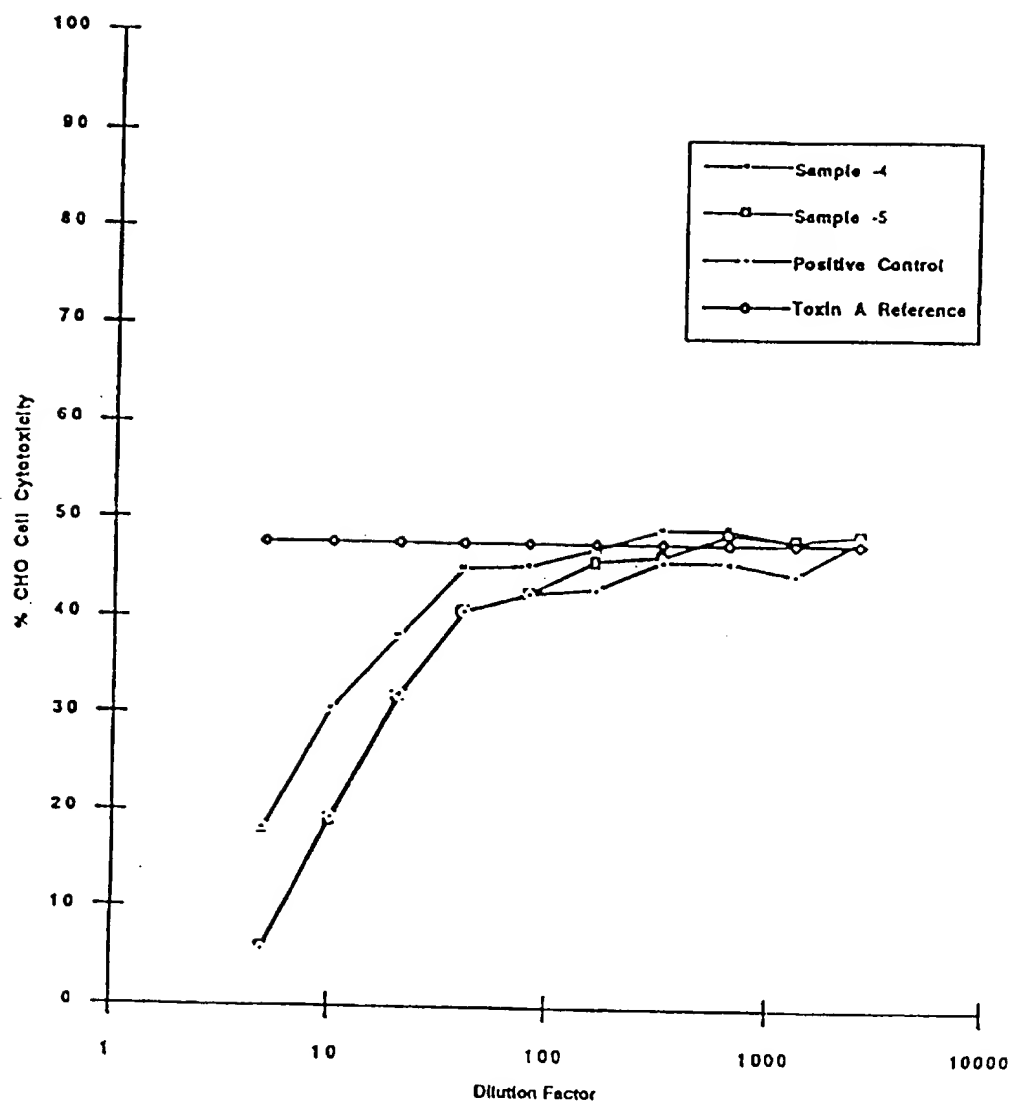




FIGURE 12



12/40

FIGURE 13

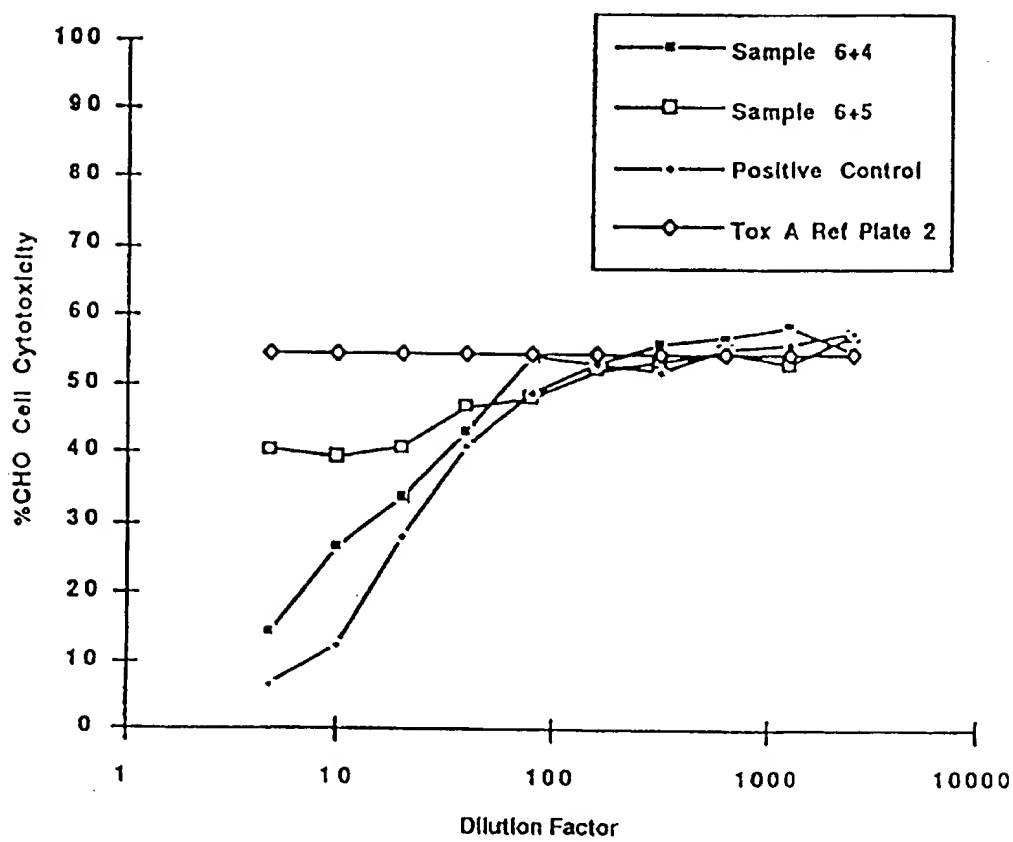


FIGURE 14

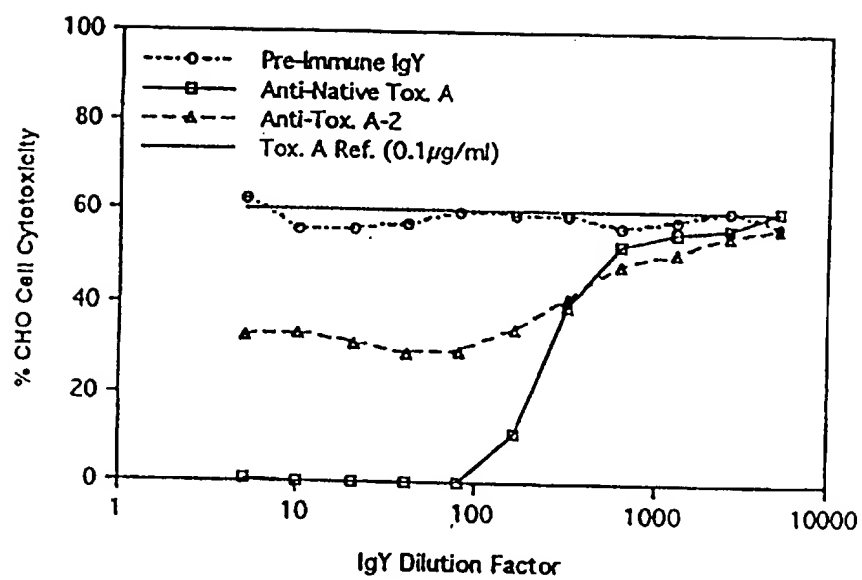
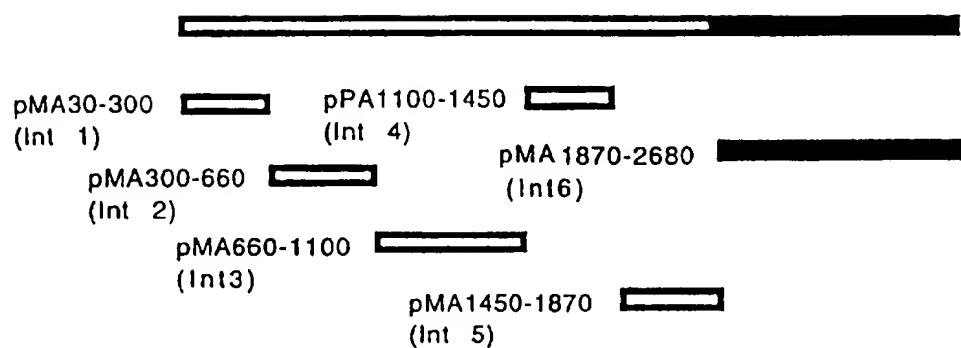


FIGURE 15

A



B

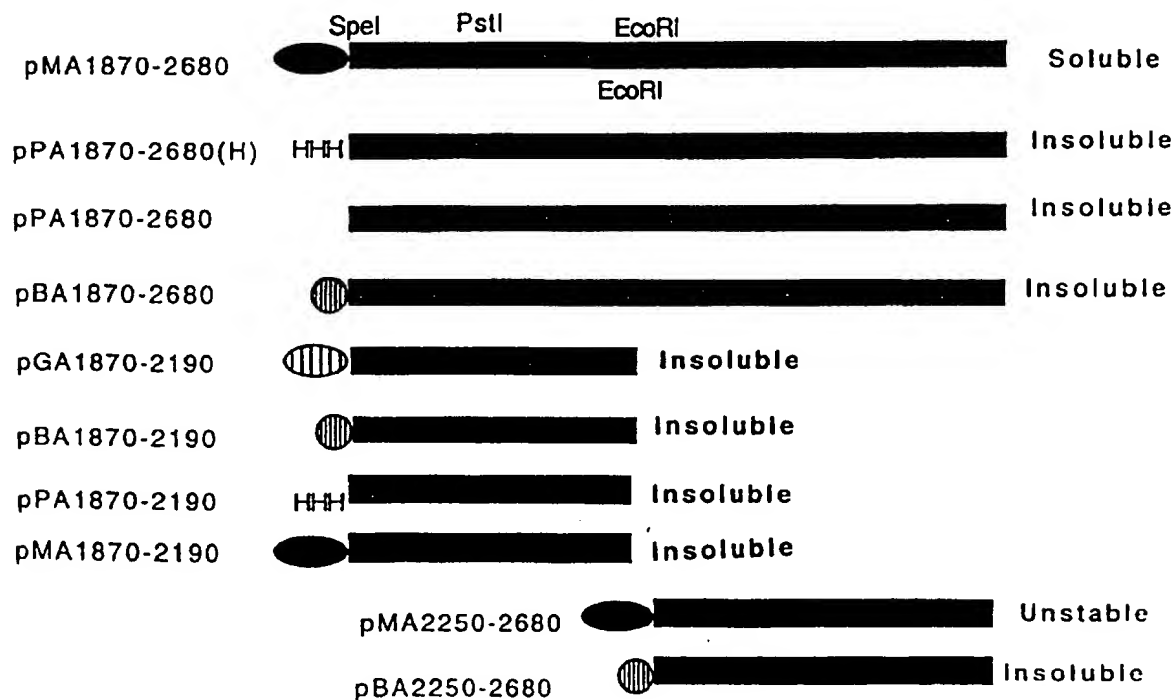


FIGURE 16

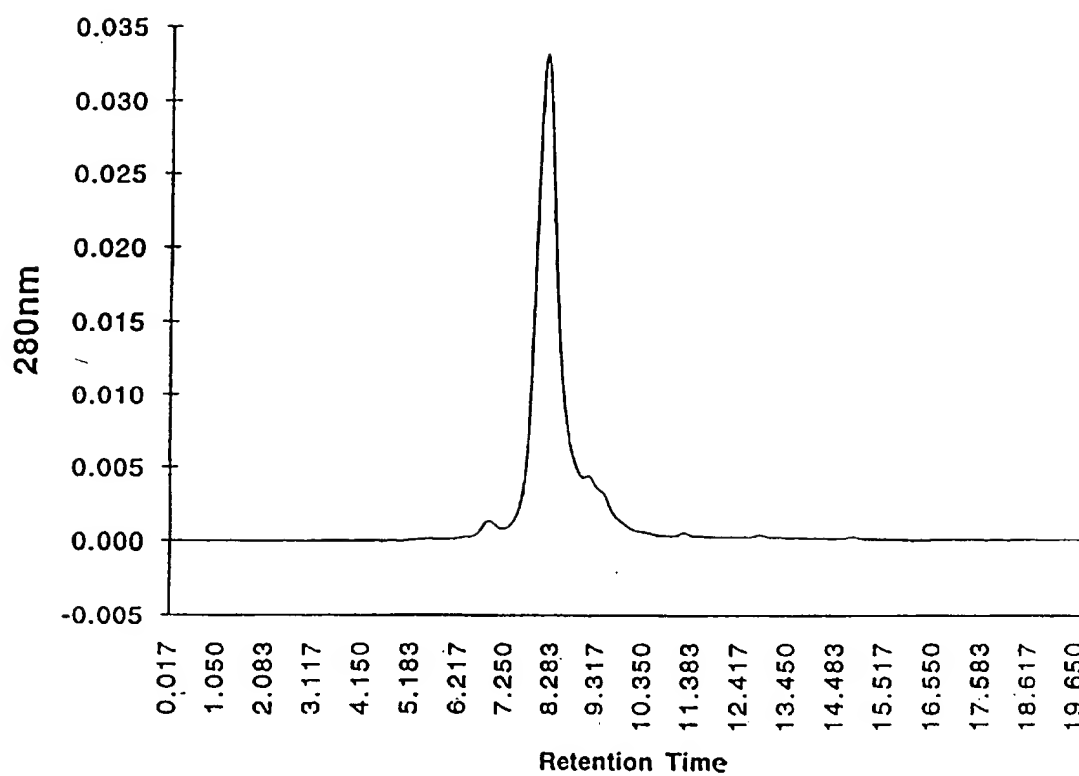


FIGURE 17

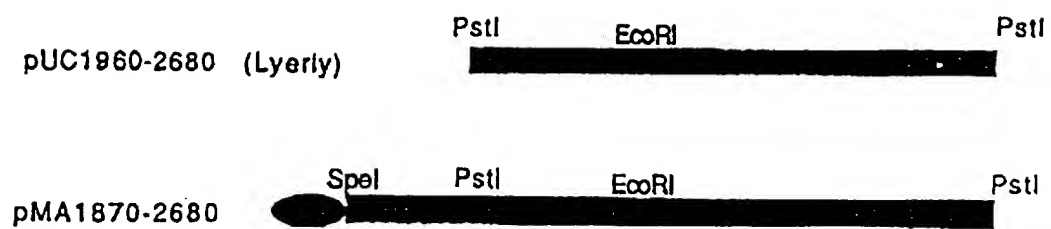


FIGURE 18

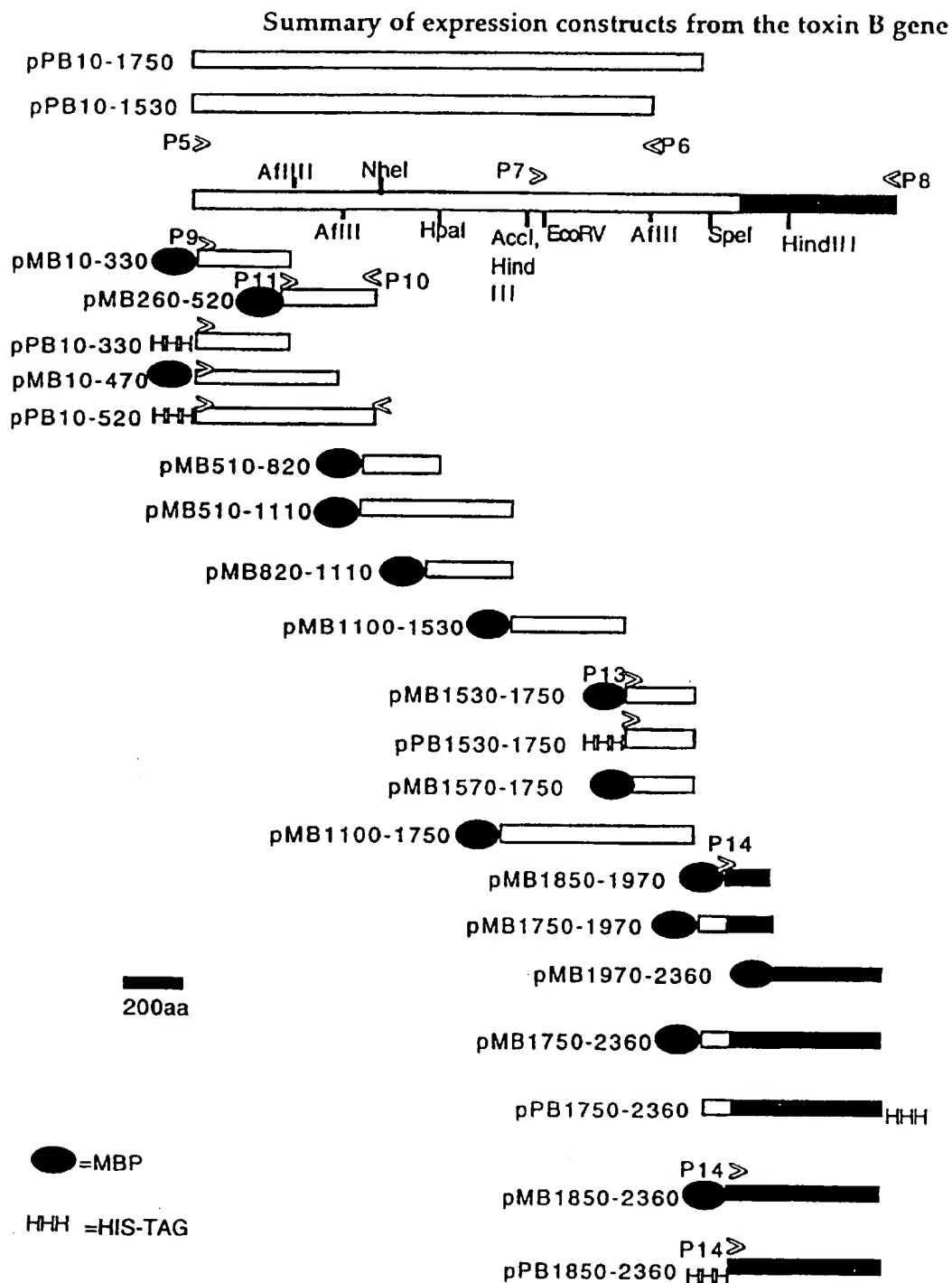


FIGURE 19

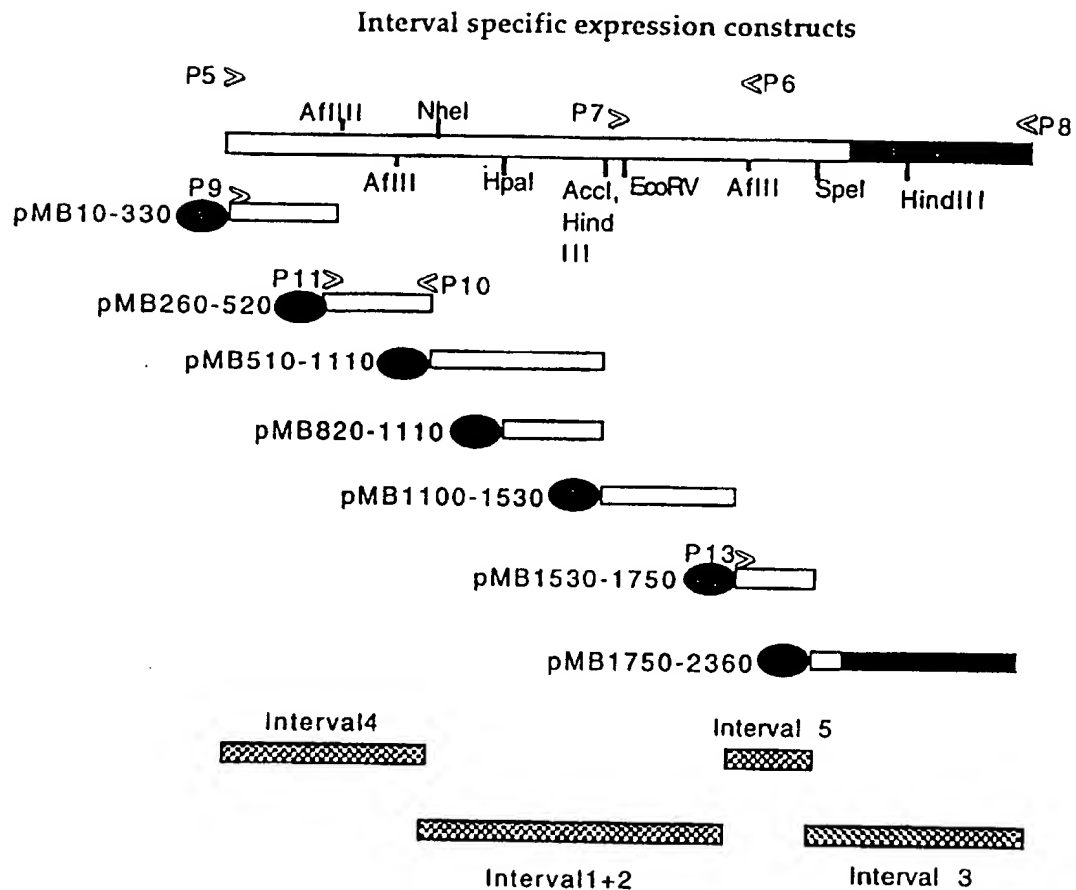




FIGURE 20

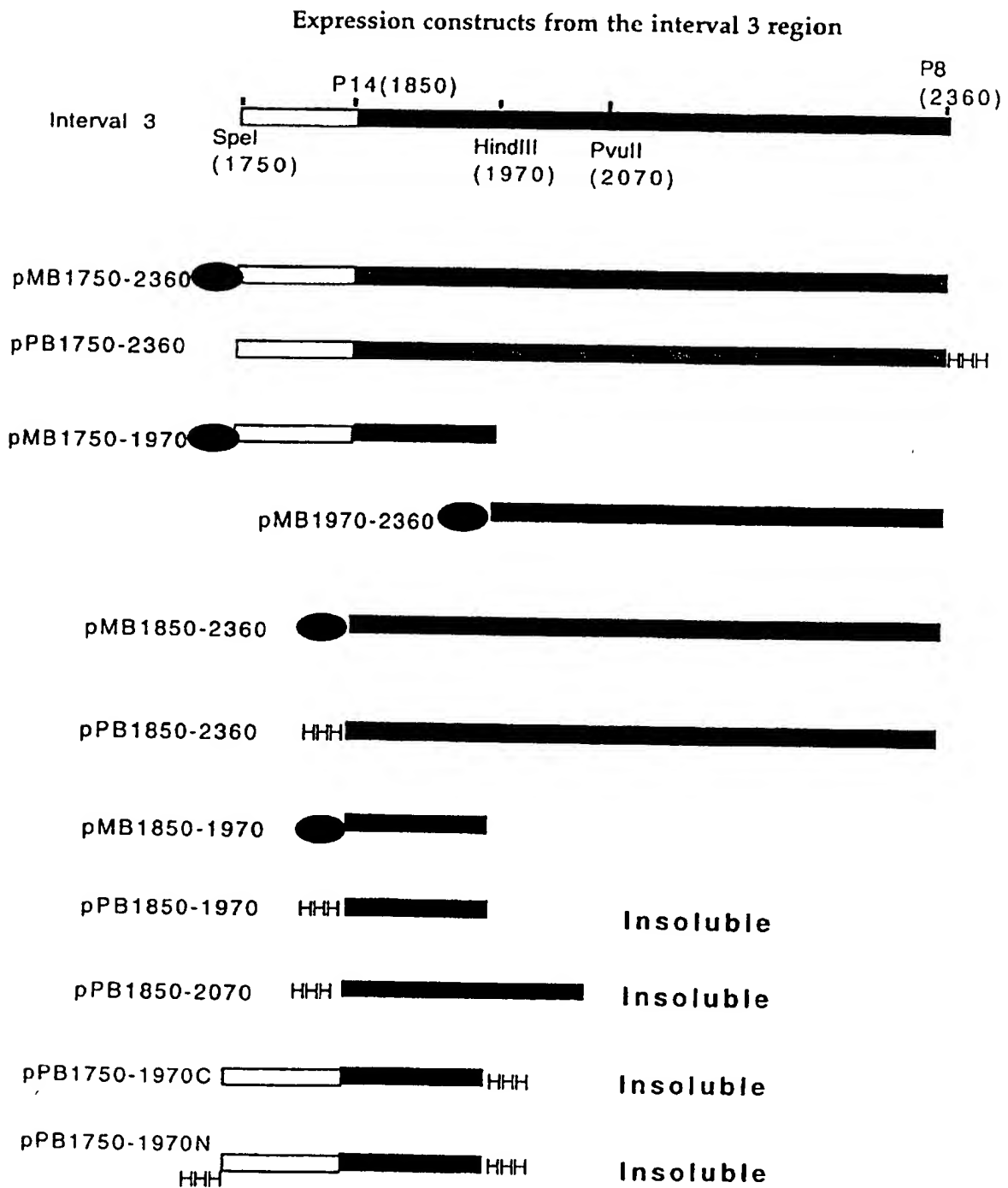


FIGURE 21

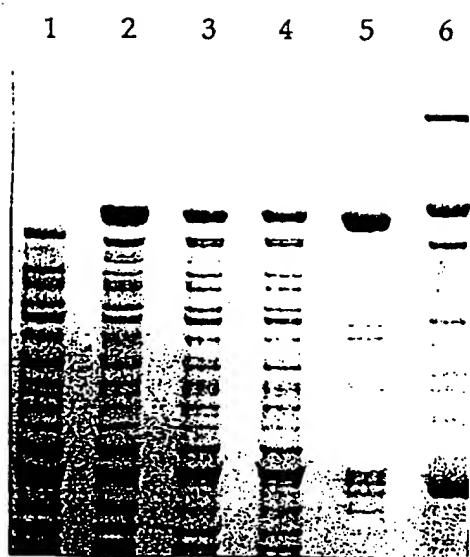


FIGURE 22

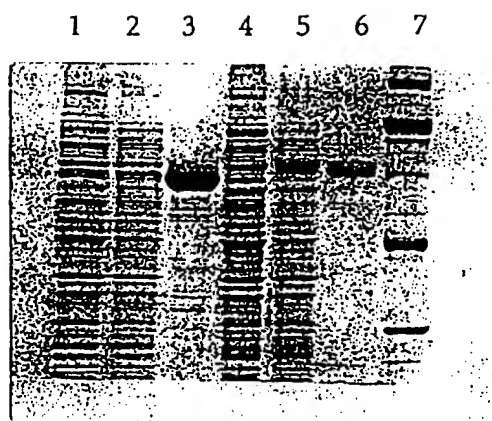


FIGURE 23

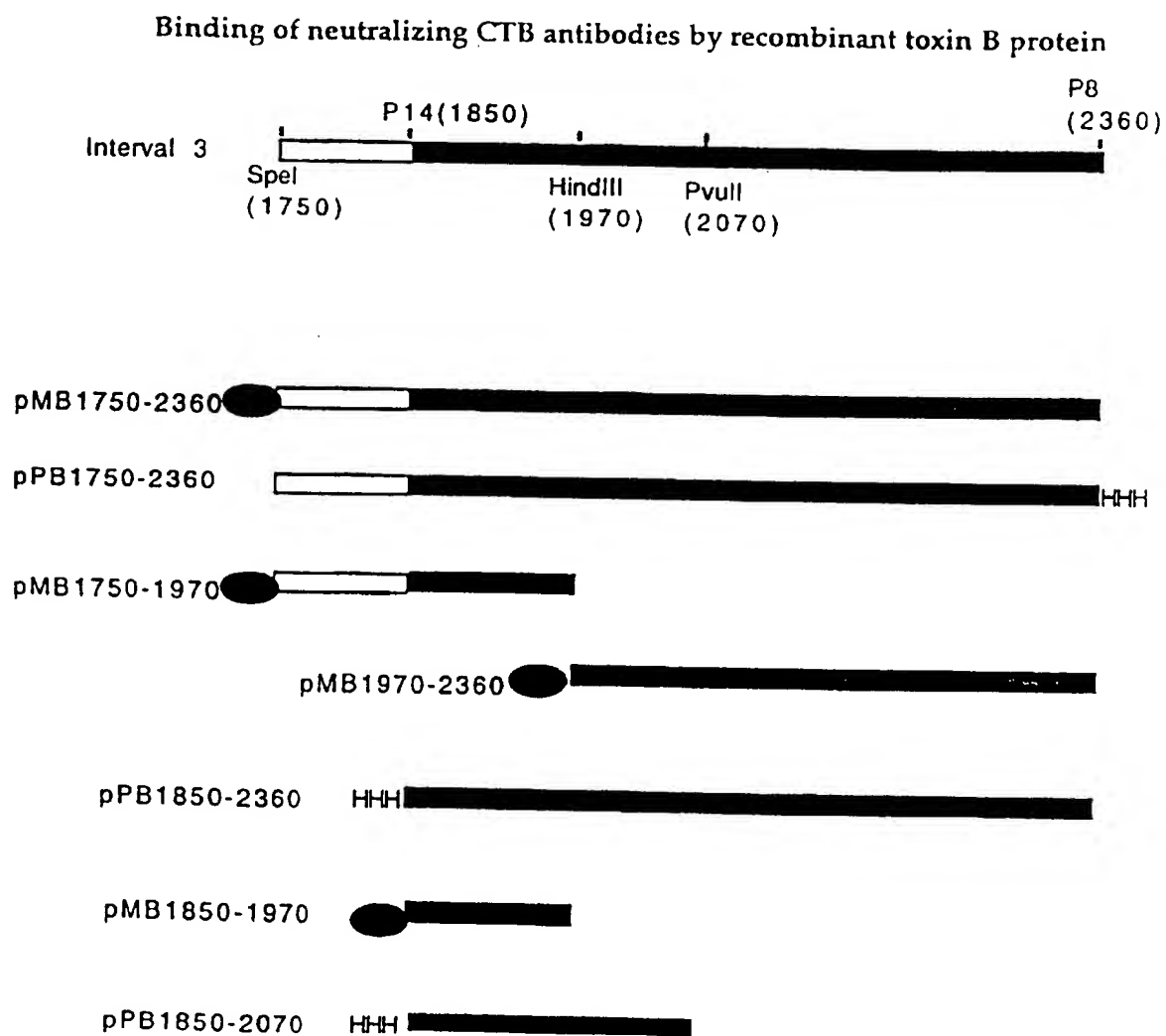


FIGURE 24

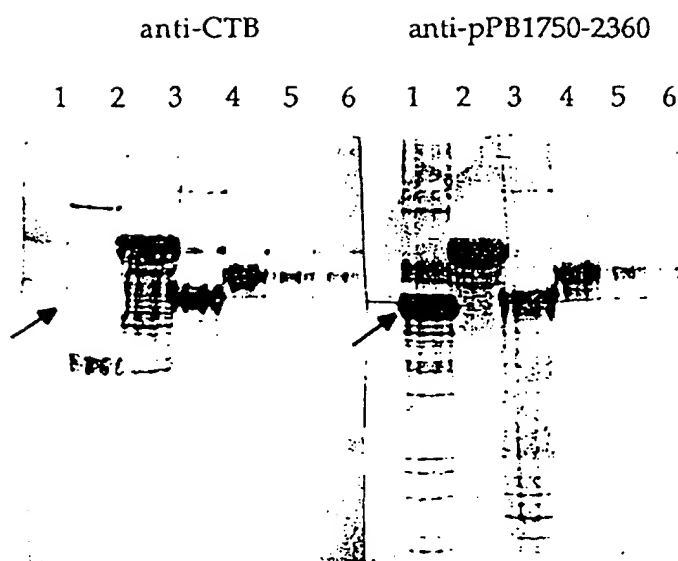


FIGURE 25

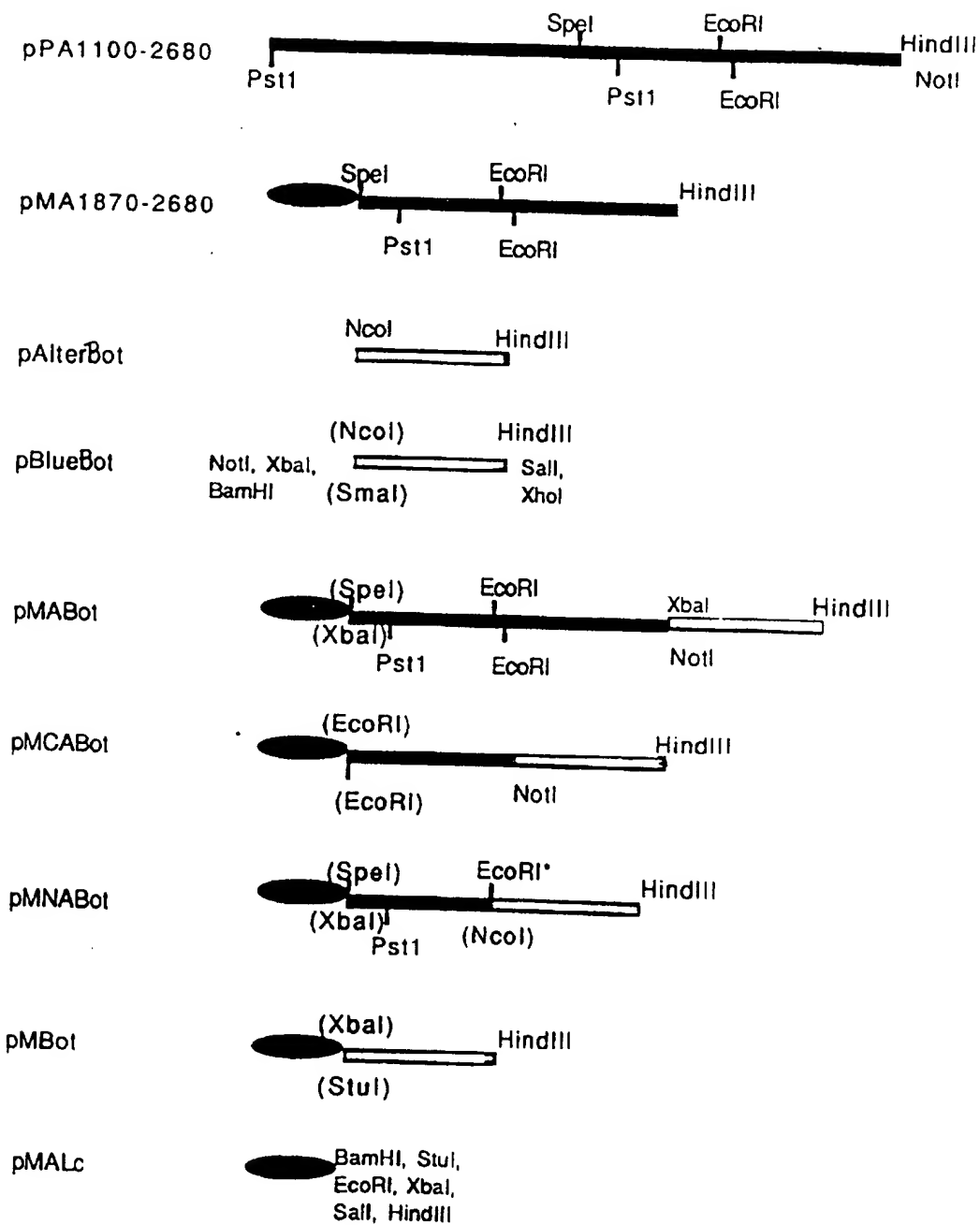
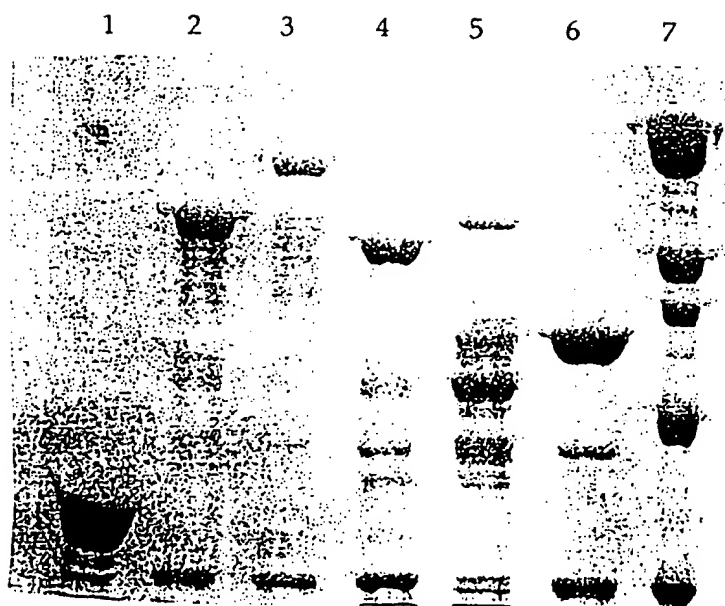


FIGURE 26



## FIGURE 27

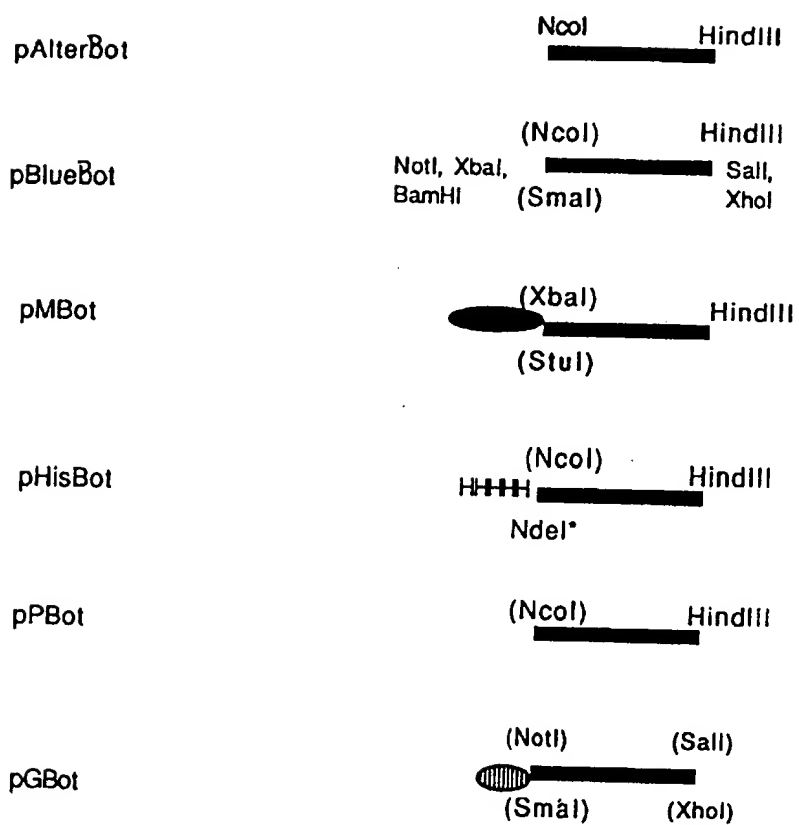




FIGURE 28

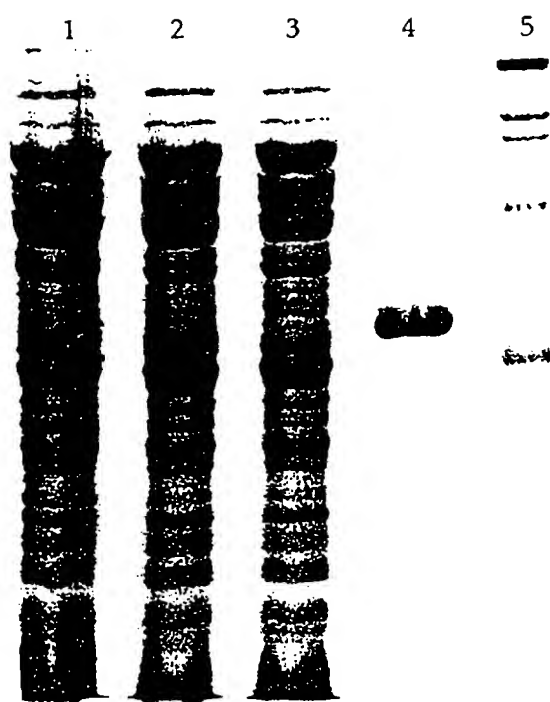


FIGURE 29

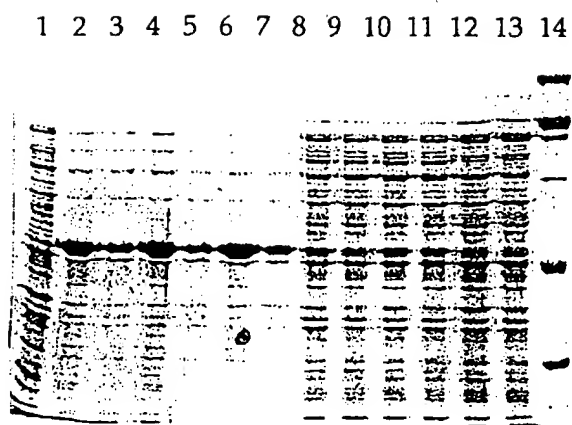


FIGURE 30

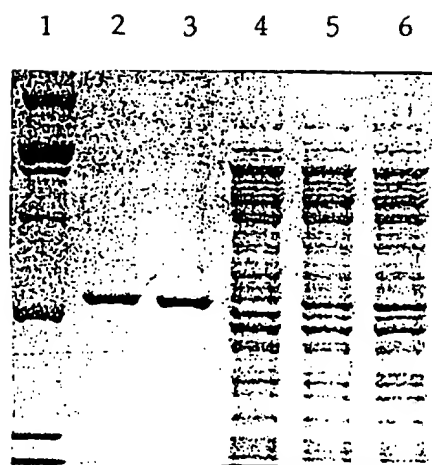


FIGURE 31

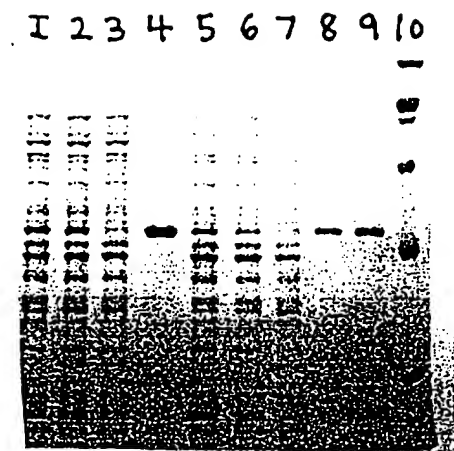


FIGURE 32

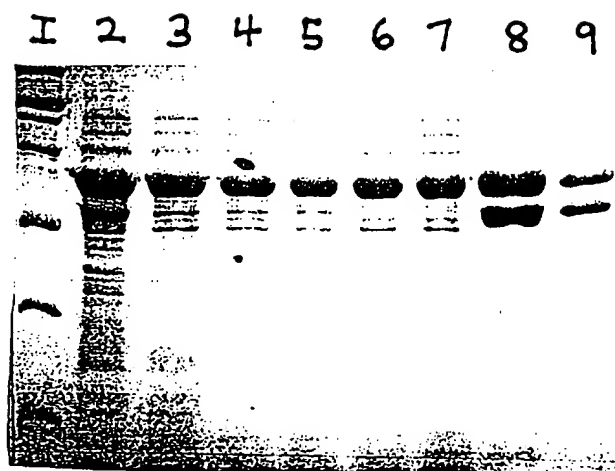


FIGURE 33

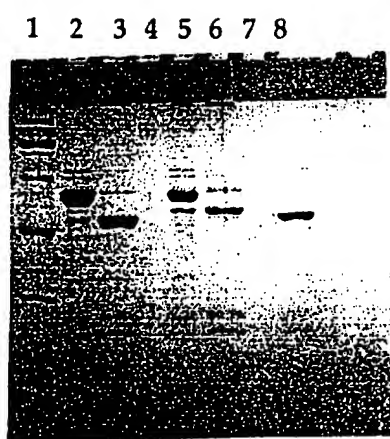


FIGURE 34

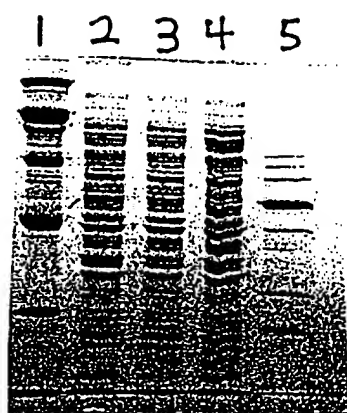


FIGURE 35

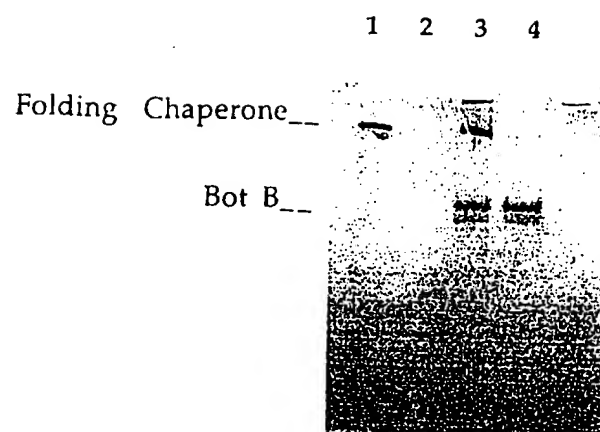




FIGURE 36

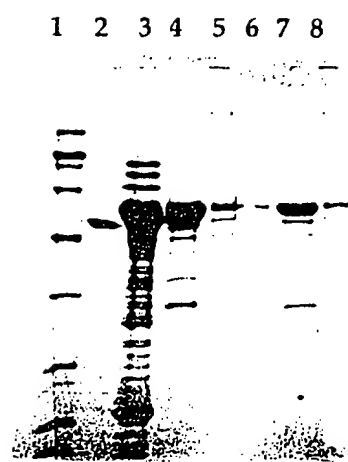


FIGURE 37

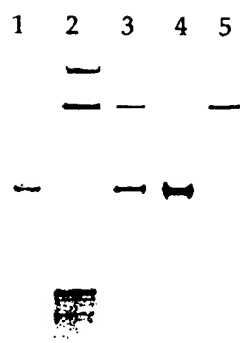


FIGURE 38

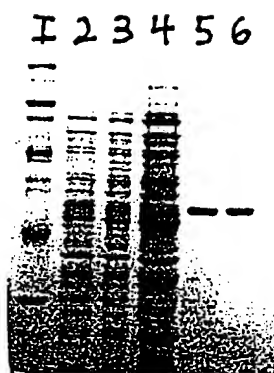


FIGURE 39

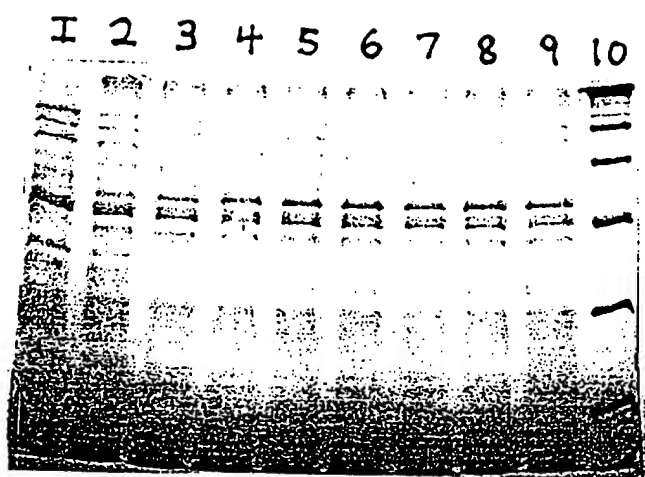
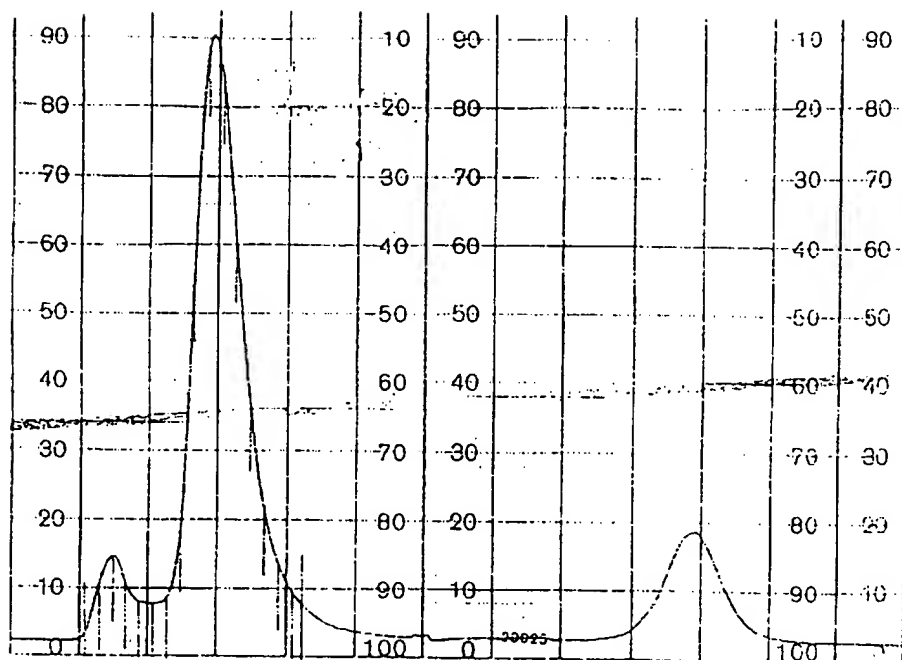


FIGURE 40



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/JS97/15394

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : Please See Extra Sheet.

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/184.1, 192.1, 247.1; 435/69.1, , 69.7, 325, 320.1; 530/388.4, 389.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS, WPIDS, CAPLUS, APS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	THOMPSON et al. The Complete Amino Acid Sequence of the Clostridium botulinum Type A Neurotoxin, Deduced by Nucleotide Sequence Analysis of the Encoding Gene. Eur. J. Biochem. April 1990, Vol. 189, pages 73-81, see entire document.	1-24
Y	BINZ et al. The Complete Sequence of Botulinum Neurotoxin Type A and Comparison with Other Clostridial Neurotoxins. Journal of Biological Chemistry. June 1990, Vol. 265, No. 16, pages 9153-9158, see entire document.	1-24
Y	ROITT. Essential Immunology. Oxford: Blackwell Scientific Publications. 1988, especially pages 173-178.	1-24



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

10 OCTOBER 1997

Date of mailing of the international search report

23 DEC 1997

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

EVELYN RABIN

Telephone No. (703) 308-0196

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US97/15394

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SIEGEL. Human Immune Response to Botulinum Pentavalent (ABCDE) Toxoid Determined by a Neutralization Test and by an Enzyme-Linked Immunosorbent Assay. Journal of Clinical Microbiology. November 1988, Vol. 26, pages 2351-2356, see entire document.	1-24
Y	FORD et al. Fusion Tails for the Recovery and Purification of Recombinant Proteins. Protein Expression Purification. 1991, Vol. 2, pages 95-107, see entire document.	1-24
Y	LECLERC et al. Induction of Virus-Neutralizing Antibodies by Bacteria Expressing the C3 Poliovirus Epitope in the Periplasm. Journal of Immunology. April 1990, Vol. 144, pages 3174-3182, see entire document.	1-24
Y	KLEID. Using Genetically Engineered Bacteria for Vaccine Production. Annals New York Acad. Sci. 1983, Vol. 483, pages 23-30, see entire document.	1-24

**INTERNATIONAL SEARCH REPORT**

International application No.  
PCT/JS97/15394

**A. CLASSIFICATION OF SUBJECT MATTER:**

IPC (6):

A61K 39/00, 39/38, 38/08; C12P 21/06, 21/04, 21/08; C12N 15/00, 15/09, 15/63, 15/70, 15/74; C07K 16/00

**A. CLASSIFICATION OF SUBJECT MATTER:**

US CL :

424/184.1, 192.1, 247.1; 435/69.1, . 69.7, 325, 320.1; 530/388.4, 389.5